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Semliki Forest virus infection of mosquito cells -novel insights into host responses and antiviral immunity

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Declaration

I declare that all the work included in this thesis is my own except where otherwise stated. No part of this work has been, or will be submitted for any other degree or professional qualification.

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Abbreviations

Ae.aegypti	*Aedes aegypti*
Ae.albopictus	*Aedes albopictus*
AGO	Argonaute
An.gambiae	*Anopheles gambiae*
AP	Alkaline phosphate
BHK-21	Baby hamster kidney cells
BSA	Bovine serum albumin
CDC	Centers for Diseases Control and Prevention
cDNA	complementary DNA
CHIKV	Chikungunya virus
CPE	Cytopathic effect
CpG	Cytosine-phosphate-guanine
CPV	Cytoplasmic vacuoles
DCV	Drosophila C virus
DXV	Drosophila X virus
DCL	Dicer-like proteins
Dcr	Dicer
DD	Death domain
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxy-nucleoside triphosphate
dsDNA	Double-stranded deoxyribonucleic acid
dsRBD	dsRNA binding domain
dsRNA	Double-stranded ribonucleic acid
DTT	Dithiothreitol

E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EEE	Eastern equine encephalitis
EEEV	Eastern Equine Encephalitis Virus
Egf1.0	Epidermal growth factor-like 1.0
eGFP	Enhanced green fluorescent protein
eIF2α	Eukaryotic transcription initiation factor 2α
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated death domain protein
FCS	Foetal Calf Serum
FMDV	Foot-and-mouth disease virus
GMEM	Glasgow's minimal essential media
GTP	Guanosine triphosphate
H_2O	Water
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICAD	Inhibitor of caspase-activated deoxyribonuclease
IFN	Interferon
IκB	Inhibitor of kappa B
IKK	Inhibitor of kappa B kinase
IMD	Immune deficiency
IRAK	Interleukin-receptor associated kinase
IRF	IFN regulatory factor
ISGF	Interferon-stimulated gene factor
JAK	Janus family tyrosine kinase
JEV	Japanese encephalitis virus
LACV	La Crosse virus
LB	Luria-Bertani
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MAPK	Mitogen-activated protein kinase
MDA5	Melanoma differentiation-associated gene 5
MOI	Multiplicity of Infection
MyD88	Myeloid differentiation-88

NBCS	Newborn calf serum
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nsP	Non-structural protein
ONNV	O'nyong-nyong virus
ORF	Open reading frame
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PBSA	Phosphate buffered saline albumin
PFU	Plaque forming unit
PGN	Peptidoglycan
PGRP	Peptidoglycan recognition proteins
PKR	Protein kinase R
PO	Phenoloxidase
PPO	Prophenoloxidase
PRR	Pathogen recognition receptor
PTGS	Post-transcriptional gene silencing
RIG-I	Retinoic acid-inducible gene-I
RIP	Receptor interacting protein
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RRV	Ross river virus
RT	Room temperature
RT-PCR	Reverse-transcription polymerase chain reaction
RVFV	Rift Valley fever virus
SFV	Semliki Forest Virus
siRNA	Small interfering RNAs
SLEV	St. Louis encephalitis virus
SOC medium	Super Optimal broth with Catabolite repression
SPBS	Sterile phosphate buffered saline
ssRNA	Single-stranded ribonucleic acid
STAT S	Signal transducers and activators of transduction
TBE	Tris-borate EDTA
TGF	Transforming growth factor

TIR	Toll/IL-1R
TIRAP	TIR domain containing adaptor protein
TISC	Toll-induced signalling complex
TLR	Toll-Like Receptor
UV	Ultra-Violet
VEEV	Venezuelan Equine Encephalitis Virus
VRP	Viral Replicon Particles
WEEV	Western Equine Encephalitis Virus
WNV	West Nile virus

Abstract

Arboviruses are transmitted between vertebrate hosts by arthropod vectors, such as mosquitoes or ticks. In vertebrates arboviruses cause cytopathic effects and disease, however, arbovirus infection of arthropods usually results in persistence. Control of arboviral infection is mediated by the arthropod's immune system. Pathways such as RNAi, JAK/STAT, Toll and IMD have previously been implicated in controlling arbovirus infections. In contrast, the antiviral role of other pathways in mosquitoes, such as melanisation, is unknown. Using high through output 454 sequencing the transcriptome of U4.4 cells infected with the model arbovirus Semliki Forest virus (SFV)(*Togaviridae, Alphavirus*) was generated. This experiment revealed intriguing patterns of differential transcript abundance that suggest a broad impact of SFV infection in U4.4 cells, such as in metabolism, cell structure and nucleic acid processing. SFV infection induces differential expression of genes in pathways such as apoptosis, stress response and cell cycle. Most interestingly, this study indicated that melanisation might have an antiviral role in mosquitoes. In arthropods, melanisation is a process involved in wound healing and antimicrobial defences. Phenoloxidase (PO), a key enzyme involved in melanisation, is cytotoxic and therefore kept in its inactive form, prophenoloxidase (PPO), until activation is triggered. The PPO activation process is tightly regulated by serine protease inhibitors (serpins) which inhibit the proteolytic activation reaction. In this thesis I demonstrate that the supernatant of cultured *Aedes albopictus*-derived U4.4 cells contains a functional proPO-activating system, which is activated by infection with bacteria and virions of SFV. Activation of this pathway reduces the spread and infectivity of SFV *in vitro* and *in vivo*. In order to further characterise the PO cascade and its antiviral role the serpins in *Ae. albopictus* were also investigated. Using the transcriptome sequencing and bioinformatics we identified and classified 11 serpins. We silenced each of the serpins and monitored PPO levels and antiviral activity showing that homologues to drosophila's serpin-27a plays a role in melanisation against SFV in vitro. Collectively, these results characterise the mosquito PO cascade as a novel immune defence against arbovirus infection in mosquitoes.

Chapter 1: Introduction

Arboviruses, definition, classification and importance.

Arthropod-borne viruses, or arboviruses, are transmitted among vertebrate hosts by haematophagous, or blood feeding, arthropod vectors, such as mosquitoes, midges or ticks. Due to this complex multi-host cycle, arboviruses must be able to replicate in the vertebrate host as well as in the arthropod vector. Both hosts and their immune systems present several barriers that the virus has to overcome. By definition arboviruses are not just mechanically transmitted by the vector through contaminated mouth parts, but must undergo replication in the vector in order to be transferred (NicholWeaver, 1997). There are a number of different ways in which the arbovirus can be transmitted among vectors. The most common form of transmission is horizontally through a blood meal from an infected vector via the salivary glands to a vertebrate host which then becomes viraemic and allows a second vector to become infected when taking a blood meal from it. In this case the virus from the blood meal has to be able to infect the midgut, then disseminate within the vector and reach the salivary glands. It must then replicate at higher titres and be exported with the saliva during the next blood feeding (Weaver and Reisen, 2010). As will be discussed later, the arthropod immune system plays an important role in controlling replication and the motility of the virus in the vector. Other manners of transmission that are not that common, but should still be considered important are; vertical transmission, from an infected female to male and female offspring, and venereal horizontal transmission, from a vertically infected male to a female vector. Vertical and venereal transmission of arboviruses exclusively among vectors seems to be a strategy for the virus to overwinter (Goddard et al., 2003), however, it comes at a fitness cost for both the virus and the vector (Lambrechts and Scott, 2009).

Taxonomically, arboviruses include a wide variety of RNA viruses belonging to the *Togaviridae*, *Bunyaviridae*, *Flaviviridae*, *Reoviridae*, *Orthomyxoviridae* and *Rhabdoviridae* families (Fenner and Maurin, 1976; Melnick, 1976; Schmaljohn and McClain, 1996; van Regenmortel et al., 2000). Having very distinct RNA genomes and replication strategies, these viruses appear to have arisen many times during the evolution of RNA viruses. Only one DNA arbovirus is known, African Swine fever virus (ASFV) (*Asfaviridae*, genus *Asfavirus*) (Kemp et al., 1988; van Regenmortel et al., 2000). The lack of DNA arboviruses seems to correlate with the lower genetic plasticity and lower mutation rates present in RNA

viruses, restricting their ability to replicate in physiologically different hosts (Holland and Domingo, 1998).

Arboviruses circulate in the wild among vertebrate populations which are adapted to them, to a certain extent, and infection causes only some febrile illness during the period of high viraemia required for transmission and they recover later. However, in case of spillover transmission to humans and/or domestic animals, they cause disease in what are dead-end hosts. Viruses like dengue (DENV) and chikungunya (CHIKV) produce extensive epidemics since they have lost the need for an enzootic cycle. Therefore, arboviruses are a major current public health and veterinary issue worldwide. On top of that, arboviral outbreaks are dynamic, extending beyond their traditional warmer geographical climates into temperate areas. The ability of these viruses to cause epidemics and disease depends of a wide range of factors; from viral genetics to vector competence and epidemiology (Weaver and Reisen, 2010). As was previously mentioned, the vector displays several barriers which can restrict the virus by reducing competency and transmissibility, and the vector immune system plays a key role in the virus transmission cycle, infectivity and vector permissibility.

Arbovirus ecological cycles

Arboviral diseases are generally zoonotic, occurring in populations of wild animals and then spilling over into a rural or urban environment where they cause diseases in domestic animals and humans. Generally these two hosts are considered dead-end hosts since viraemia is not high enough to start a new cycle in that urban area, although, there are exceptions such as DENV, CHIKV and YFV which can cause epidemics by human to human transmission. DENV is the arbovirus with a highest public health impact worldwide (www.WHO.org). In order to describe how these two ecological cycles occur, DENV will be taken as an example as it has a sylvatic cycle and an urban cycle (Fig.1.1).

There are four DENV serotypes, and each of them is maintained in two evolutionary and ecologically different transmission cycles; a sylvatic cycle and an urban cycle. The sylvatic cycle involves non-human primates and arboreal aedine mosquitoes. It has been documented in transmission foci in forest areas of West Africa and the Malay Peninsula. The urban cycle involves the domestified *Aedes aegypti* subsp. *aegypti* and *Ae. albopictus* mosquitoes and can be found in a diverse range of environments, from cities to rural areas, throughout the tropics and subtropics (Mattingly, 1957; Mattingly, 1958). In the human cycle, humans act as the only known reservoir hosts and amplification hosts, a unique host usage pattern among arboviruses (Weaver and Reisen, 2010). The transmission of DENV between the sylvatic and the urban cycle occurs in a transitional rural environment. Arboreal *Aedes* mosquitoes are not adapted to live in urban areas, therefore, this transitional rural area also provides the opportunity for DENV to move into domestified *Aedes* mosquitoes. It has been suggested that, in the case of DENV cycles, *Ae. furcifer* carries the virus from the sylvatic to the rural environment, where it coexists with the urban *Ae. albopictus*. Once DENV is in this rural area, *Ae. albopictus* can bring the virus to the rural transition area and the urban cycle (Weaver and Reisen, 2010). It is suggested that the same occurs with other epidemic arboviruses, such as with YFV and CHIKV. It has become increasingly clear that of all the viruses with the potential to shift from an animal reservoir into humans, the most likely to shift are those, like sylvatic DENV, that are carried by our closest relatives, the non-human primates (Vasilakis et al., 2011).

Several factors control the outcome of these ecological cycles as well as the emergence of new arboviruses and vectors. These factors, such as vector permissibility, viral evolution and ecological and climatic changes will be discussed later in this thesis.

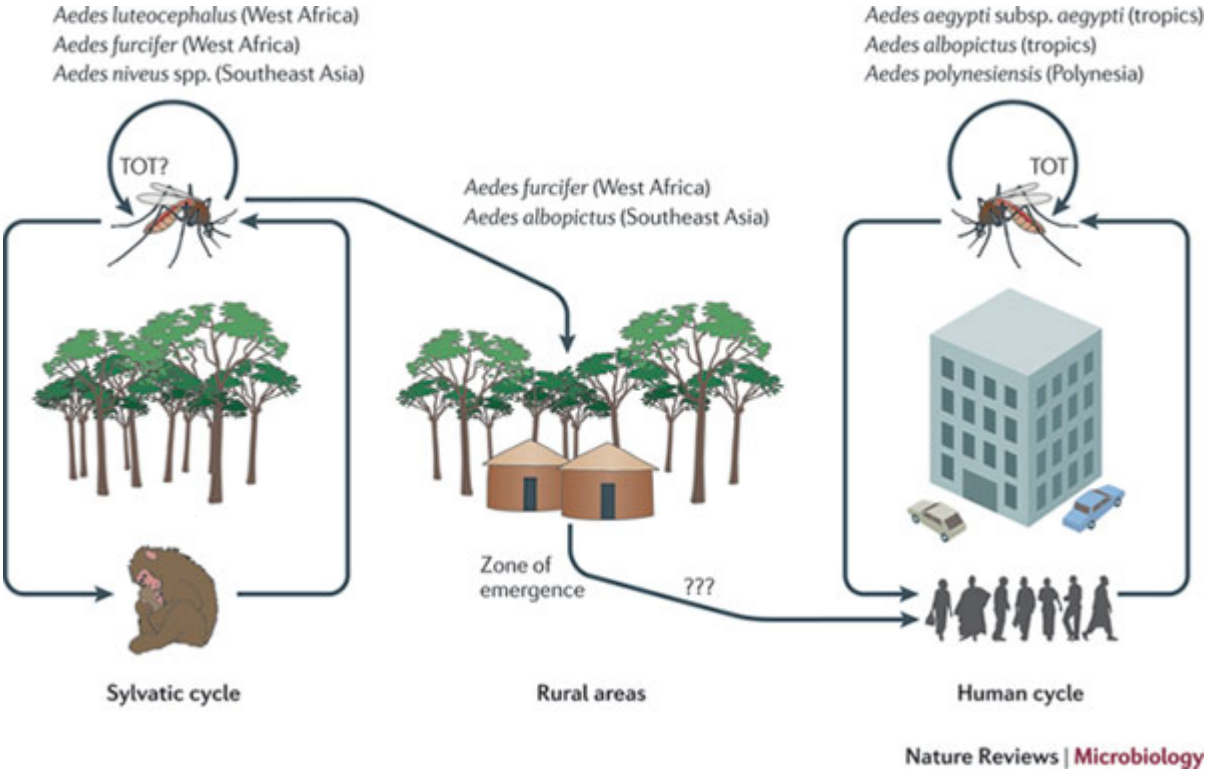

Fig.1.1. Dengue virus sylvatic and urban cycles. Dengue virus is transmitted by a set of aedine mosquitoes in its sylvatic cycle, where it circulates among small vertebrates and non-human primates. In the urban cycle it is transmitted among humans by another set of mosquitoes. Transition between both cycles occurs in rural areas and it is produced by linked *Aedes* mosquitoes, such as *Ae. furcifer* and *Ae. albopictus* which are present in sylvatic and urban areas respectively. Adapted from (Vasilakis et al., 2011).

Medically important arboviruses families

In the next section, three of the most important arbovirus families will be described in detail. Bunyaviruses, flaviviruses, orbiviruses and alphaviruses count for most of the important human and veterinary arboviral diseases.

Bunyaviridae

Bunyaviruses form the largest family of RNA viruses with over 350 isolates. These viruses are enveloped and their virions are spherical, around 100 nm in diameter. The most characteristic property is the possession of a negative (or ambisense) single stranded RNA genome divided into three segments (Nichol, Beaty, and Elliott, 2005). Bunyavirus virions are formed by four structural proteins, two of them are external glycoproteins (Gn and Gc) and they are encoded by the M (medium) segment of the genome; the nucleocapsid, N, which is encoded by the S (small) segment, coats the genomic RNA; and the final protein is the RNA dependent RNA polymerase, which is encoded by the L (large) segment of the genome (Elliott, 2009). This large virus family is divided into five genera: *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus* and *Tospovirus*, based on serological relationships as well as a characteristic conserved patterns of the segments of the RNA genome (Nichol, Beaty, and Elliott, 2005). Hantaviruses are viral zoonotic pathogens of rodents and can cause severe human disease, such as haemorrhagic fevers. Tospoviruses are plant pathogens transmitted by sap feeding arthropods. Orthobunyaviruses, nairoviruses and phleboviruses are true arboviruses transmitted among vertebrates by haematophagus arthropods, like mosquitoes, midges, sandflies and ticks (Elliott, 2009).

Several bunyaviruses transmitted by vectors cause human or veterinary diseases (Table 1.1). The phlebovirus Rift Valley fever virus (RVFV) causes epizoonotic and sporadic epidemics, primary in east Africa (Bird et al., 2009). Infection can cause up to 10% fatality in sheep and cattle spilling occasionally to humans, in whom it causes severe disease in 1-2% of cases. Severe disease includes hepatitis, encephalitis, renitis, blindness, haemorrhagic fever and case fatality is 10-20% (Madani et al., 2003). RVFV was endemic to the Rift Valley, in Kenya and Tanzania, where outbreaks by the various lineages of this virus were common (Bird et al., 2008). However, in the second half of last century, outbreaks started appearing in other parts of the African continent, such as in the 1950s in South Africa, in the late 1970s in Egypt, in the late 1980s in Mauritania and the Maghreb, during the 1990s in Madagascar and in the

new millennium it crossed into the Arabian Peninsula (Bird et al., 2009). Emergence of RVFV has been correlated with weather conditions and the spread by diverse *Aedes* species mosquitoes (Anyamba et al., 2009; Linthicum et al., 1985).

Genus/virus	Disease	Principal Vector	Geographical distribution
Orthobunyavirus			
Akabane	Cattle: abortion and congenital defects	Midge	Africa, Asia, Australia
Cache Valley	Sheep, cattle, congenital disorders	Mosquito	North America
La Crosse	Human: encephalitis	Mosquito	North America
Ngari	Human: haemorrhagic fever	Mosquito	Africa
Oropouche	Human: fever	Midge	South America
Tahyna	Human: fever	Mosquito	Europe
Nairovirus			
Crimean-Congo haemorrhagic fever	Human: haemorrhagic fever	Tick	Eastern Europe, Africa, Asia
Nairobi sheep disease	Sheep, goat: fever, haemorrhagic gastroenteritis, abortion	Tick, mosquito	Africa, Asia
Phlebovirus			
Rift Valley Fever	Human: encephalitis, haemorrhagic fever, renitis. Domestic ruminants: necrotic hepatitis, haemorrhage, abortion	Mosquito	Africa
Naples sandfly fever	Human: fever	Sandfly	Europe, Africa
Sicilian sandfly fever	Human fever	Sandfly	Europe, Africa

Table 1.1. Arboviruses belonging to the Bunyaviridae family. Pathogenesis, vectors and distribution.

Flaviviridae

The genus *Flavivirus* in the *Flaviviridae* family includes arboviruses transmitted between vertebrate hosts by mosquitoes and ticks producing disease in humans and animals (see Table 1.2). They take their name from yellow fever virus (*flavus* = yellow in Latin). Flaviviruses have an enveloped virion and their genome is a positive sense single stranded RNA transcript uncapped and non-polyadenylated. Instead, for translation and replication they rely on a highly structured UTR at the 5' and 3' regions of the viral genome. The genome is translated as a polyprotein that is cleaved by cellular serine proteases and viral proteases (Gould et al., 2003).

Several of the most important arboviral diseases are caused by flaviviruses (Table 1.2). Yellow fever virus causes jaundice in people who are severely affected (hence its name and the name of the whole viral family). Sporadic outbreaks occur in sub-Saharan Africa, and yearly incidence rates can reach 200 000 infections, including 30 000 deaths (Gould et al., 2003). There are fewer reported cases in the Americas, where YF also occurs, than in sub-Saharan Africa. The reasons for viral attenuation are unknown. The four DENV serotypes (1 to 4) cause more than 50 million infections yearly, with vasculopathy in 1–10% of the most severe cases (www.WHO.org). Worldwide travel of infected people, failure to control *Aedes aegypti* in epidemic areas, and commercial transportation of infected mosquitoes have contributed to the geographic dispersal and epidemic outbreaks. Dengue fever has been recognised for over 200 years, but following the 1950s, outbreaks of more severe diseases were recognised for the first time (Gould and Solomon, 2008). Japanese encephalitis virus (JEV) infects mostly *Culex* species that feed on birds, humans, pigs, horses, reptiles, or amphibia. This virus is the most important human pathogen within its phylogenetic subgroup and it has produced up to 50,000 cases of encephalitis every year with case fatality rates of about 25% (Mackenzie, Barrett, and Deubel, 2002). West Nile virus (WNV) is related to JEV and probably originated in Africa during the past millennium prior to being dispersed north and eastwards by migratory birds (Gould et al., 2003). Outbreaks of varying intensity take place occasionally in humans, birds, and horses in Africa, Europe, Russia, Australasia and recently in America (Bakonyi et al., 2005; Hubalek and Halouzka, 1999; Lanciotti, 1999). Outbreaks occur after warm humid summers that provide ideal conditions for mosquito amplification and efficient virus transmission. There is also serological evidence of a West Nile-like virus circulating in birds in the UK, but no virus has been isolated and no disease in people has been recorded, probably due to lack of local vector permissibility (Bakonyi et al.,

2005). Another virus related to JEV is St Louis encephalitis virus (SLEV), but this has only been isolated in the Americas. The virus was possibly transported from Africa on ships crossing the Atlantic (Gould et al., 2003). Epidemics affecting up to 800 people per 100 000 in the population arise every 5–15 years with overall case fatality rates about 7%. Patients are affected by age, with highest rates for the elderly. This virus asymptomatically infects birds, implying that this relationship between birds, mosquitoes, and the virus has existed for some time (Gould and Solomon, 2008). Among the tick transmitted flaviviruses, tick-borne encephalitis virus (TBEV) and its relatives can produce mild febrile illness, biphasic fever, encephalitis, and even haemorrhagic fever in humans (Gritsun, Nuttall, and Gould, 2003). They circulate fairly harmlessly in rodents and birds in forests, moorlands and Steppe regions of Europe and Asia. The virulent southeast Asian strains have been reported as far west as Latvia and Estonia, possibly carried from the east by migratory birds (Gritsun, Nuttall, and Gould, 2003).

Virus	Location of isolation	Geographic distribution	Principal vector species	Principal host species	Human disease
Alkhurma	Saudi Arabia	Arabian Peninsula?	*Ornithodorus savignyi*?	Human beings, sheep, camels	Haemorrhagic fever
Apoi	Japan	Japan	Unknown	Rodents?	Encephalitis
Bagaza	Central African Republic	Africa	*Culex* spp	Unknown	Fever
Banzi	South Africa	Africa	*Culex* spp	Unknown	Fever
Bussuquara	Brazil	Brazil	*Culex* spp	Unknown	Fever
Dakar bat	Senegal	Africa	Unknown	Bats?	Fever
Dengue 1	Hawaii	Tropics, subtropics	*Aedes aegypti*	Human beings	Fever, rash, vasculopathy
Dengue 2	New Guinea	Tropics, subtropics	*Aedes aegypti*	Human beings	Fever, rash, vasculopathy
Dengue 3	Philippines	Tropics, subtropics	*Aedes aegypti*	Human beings	Fever, rash, vasculopathy
Dengue 4	Philippines	Tropics, subtropics	*Aedes aegypti*	Human beings	Fever, rash, vasculopathy
Ilheus	Brazil	South and Central America	*Culex* spp?	Birds	Fever
Japanese encephalitis	Japan	Asia	*Culex trita-eniorhynchus*	Birds	Encephalitis
Koutango	Senegal	Senegal	Unknown	Rodents?	Fever, rash
Kyasanur Forest disease	India	India	*Haemaphysalis spinigera*	Monkeys	Haemorrhagic fever
Langat	Malaysia	Malaysia, Thailand, Siberia	*Ixodes granulatus*	Unknown	Encephalitis
Louping ill	Scotland	UK, Ireland	*Ixodes* spp	Sheep, grouse, hares	Encephalitis
Modoc	USA	USA	Unknown	*Peromyscus maniculatus*	Encephalitis
Murray Valley encephalitis	Australia	Australia, New Guinea	*Culex annulirostris*	Birds	Encephalitis
Ntaya	Uganda	Africa	Mosquitoes	Unknown	Fever
Omsk haemorrhagic fever	Russia	Western Siberia	*Dermacentor* pictus	Muskrats, rodents?	Haemorrhagic fever
Powassan	Russia, USA, Canada	Russia, USA, Canada	*Ixodes* spp	Small mammals	Encephalitis
Rio Bravo	USA	USA, Mexico	Unknown	*Tadanida braziliensis mexicana*	Fever
Rocio	Brazil	Brazil	*Culex* spp?	Birds	Encephalitis
St Louis encephalitis	USA	South and Central America	*Culex* spp	Birds	Encephalitis
Sepik	New Guinea	New Guinea	Mosquitoes	Unknown	Fever
Spondweni	South Africa	Africa	*Aedes circumluteolus*	Unknown	Fever
Tick-borne encephalitis	Russia	Europe, Asia	*Ixodes* spp	Rodents?	Encephalitis
Usutu	South Africa	Africa	Mosquitoes	Birds	Fever, rash
Wesselsbron	South Africa	Africa, Asia	*Aedes* spp	Unknown	Unknown
West Nile	Uganda	Worldwide	Mosquitoes, ticks	Birds	Encephalitis
Yellow fever	Ghana	Sub-Saharan Africa, South America	*Aedes* spp/*Haemagogus* spp	Monkeys	Pantropic
Zika	Uganda	Africa, Asia	*Aedes* spp	Monkeys?	Fever, rash

Table 1.2. Arboviruses belonging to the Flaviviridae family. Pathogenesis, vectors and distribution.

Togaviridae

The *Togaviridae* family contains two genera, the alphaviruses and the rubiviruses, however, only the alphaviruses are considered arboviruses. Currently, there are 29 known alphaviruses, each of them with several variants and strains. They are distributed around the world and grouped by geographic Old World and New World virus distribution. Alphaviruses have a large host and cell range tropism and can cause a broad range of veterinary and human diseases. In general the geographical distribution can be associated with the onset of illness produce by each of the viruses (Peters CJ, 1990) (Fig.1.2). New World alphaviruses tend to cause encephalitis whereas Old World alphaviruses tend to cause arthritis and rashes. Exceptions to this rule are Sindbis virus (SINV) and Semliki Forest virus (SFV) which can cause neurological disease in mice, and Ross river virus (RRV) and chikungunya virus (CHIKV), which cause neurological pathologies in humans (Zacks and Paessler, 2010). Alphavirus morphology and replication strategies will be discuss in the next section of this chapter. Alphaviruses are not only of interest due to their pathogenicity to humans and domestic animals, but also for their potential role as gene therapy vectors and their possible weaponization into bioterrorism agents (Lundstrom, 2003; Sidwell and Smee, 2003).

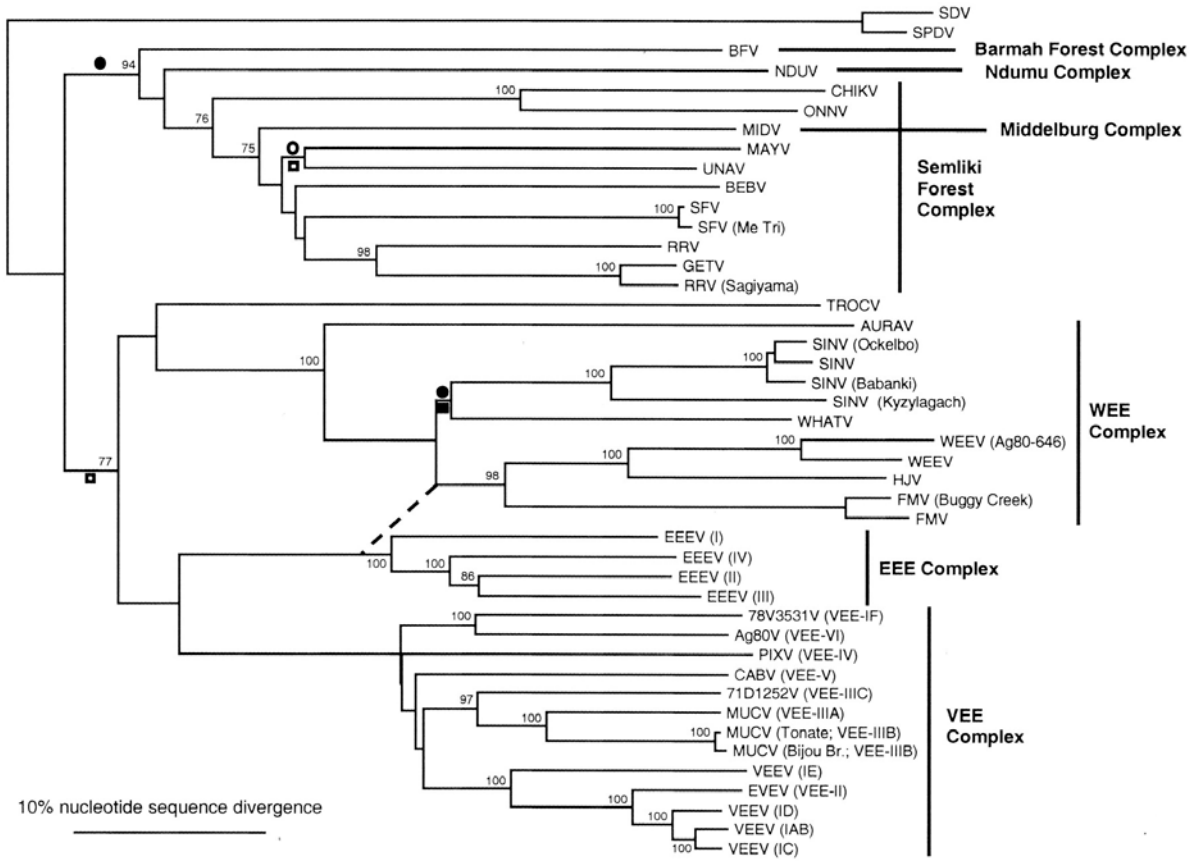

Fig.1.2. Phylogenetic tree of alphaviruses. Genetic analysis was done based on the E1 protein. New World (EEE, WEE, VEE) and Old World Complexes (SFV, SINV, CHKV) can be distinguished. Adapted from (Powers et al., 2001).

WEEV, VEEV, and EEEV can cause periodic epidemics in the Americas. The virus is maintained in the wild between rodent (WEEV and VEEV) and bird (EEEV and WEEV) reservoirs by *Culex*, *Culiseta* and *Aedes* mosquito species. Spillover to horses and humans produces epidemics in endemic countries. While all three viruses (EEEV, WEEV, and VEEV) cause encephalitis in horses and humans, their virulence and incidence vary greatly within these hosts. EEEV is the most virulent of the equine encephalitis trio with human case-fatality rates estimated to be in the range of 50% to 70% and horse case-fatality rates are estimated at 70% to 90% (Zacks and Paessler, 2010) However, EEEV has the lowest incidence of human cases of the three with only 257 confirmed human cases occurring in the United States from 1964 to 2008 (Weaver and Reisen, 2010). At the moment, there are no human vaccines approved against any of these viruses, however, there are some promising ones under development (Zacks and Paessler, 2010). Several equine vaccines exist in

endemic countries, but these do little to prevent spread since horses are not their primary vertebrate reservoirs (Hollidge, González-Scarano, and Soldan, 2010).

CHIKV is an Old World alphavirus that has re-emerged in the form of outbreaks in the last decade (Schuffenecker et al. 2006, Pialoux et al. 2007) (Fig.1.2). In Makonde language, chikungunya means “that which bends up” which describes the painful, contorted posture shown by those who are infected with this arbovirus. In its endemic African transmission cycle, CHIKV relies on wild primates and small rodents. CHIKV is transmitted by aedine species; *Ae. aegypti* and *Ae. albopictus* are the main vectors in Asia where the virus is maintained in an urban amplification cycle (Kumar et al. 2008). In the past decade, a mutation (A226V) in the viral envelope (E1) glycoprotein increased the infectivity of CHIKV for *A. albopictus* and resulted in an outbreak on the island of La Reunion in the Indian Ocean (Vazeille et al., 2007). During the 2005–2006 La Reunion outbreak, as many as 40% of the 785,000 inhabitants were infected with CHIKV. The epidemic subsequently spread to the Indian subcontinent where outbreaks involving nearly 1.5 million people occurred in India, before expanding to South East Asia, and afterwards to Europe (Charrel and de Lamballerie, 2008; Krishnamoorthy et al., 2009; Nandha and Krishnamoorthy, 2009). Worryingly, during the last decade epidemic CHIKV virulence increased causing encephalitis in some elderly and infant patients (Robin et al., 2008).

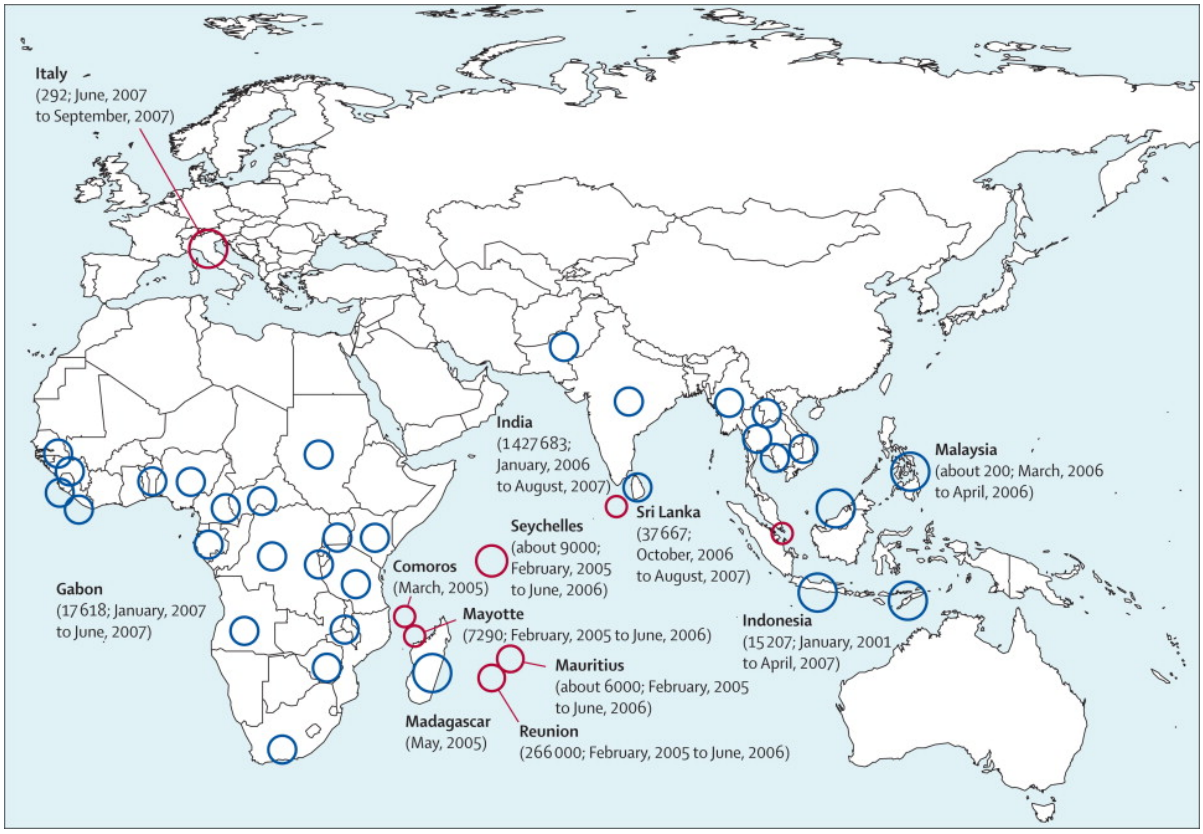

Fig.1.3. Global distribution of CHIKV. Blue circles indicate historical distribution of CHIKV in countries where the virus has been identified by virus isolation or serological evidence. Red circles indicate global re-emergence within the past decade. The number of cases, when available, is given in parentheses, along with dates of outbreak. Adapted from (Burt et al.) (www.who.org).

Alphaviruses: morphology, and replication cycle

Alphaviruses are small enveloped viruses with positive single stranded RNA genomes of around 11.5 kb that encode 9 different proteins. The genome of these viruses is divided into 2 distinct open reading frames; the larger 5' ORF takes over two thirds of the genome and contains the non-structural genes that encode the replicase (nsP1-4) The 3' ORF takes the remaining one-third of the genome and encodes the structural proteins that form the virus particle (capsid protein C, 6K, and the structural glycoproteins E1-E3) (Kaariainen et al., 1987) (Fig.1.4).

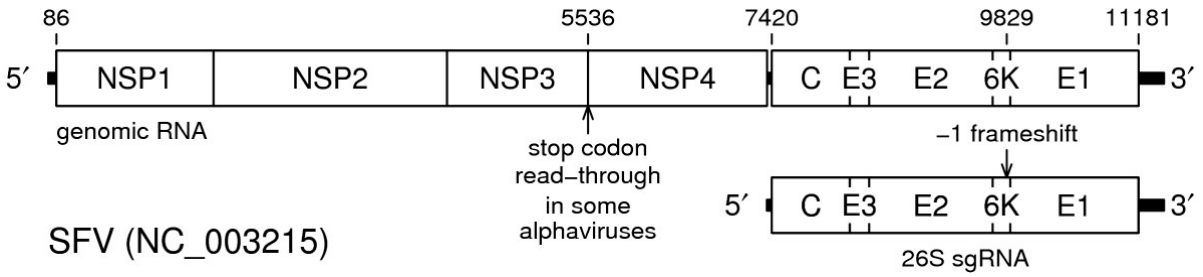

Fig.1.4. SFV genome. A typical alphavirus genome, with the replicase at the 5' end of the genome and the structural proteins under a subgenomic promoter at the 3' of the genome. The leaky stop-codon at position 5536 controls expression of the nsp4 polymerase. Adapted from (Firth et al., 2008).

Alphaviruses enter the cell by receptor mediated endocytosis (Marsh and Helenius, 1980) The alphavirus receptor is probably laminin, which is present on a great variety of vertebrate and arthropod cells (Strauss et al., 1994). Once the virus has bound, an active clathrin-dependent endocytic pathway is required for successful infection (DeTulleo and Kirchhausen, 1998). The endocytosed vesicle fuses with an endosome and then a lysosome so that the contents can be broken down (Strous and Govers, 1999). The interior of lysosomes becomes an increasingly acidic environment due to lysosome maturation. This low pH induces a conformational change in the glycoprotein spike complexes allowing the virus to fuse with the lysosome membrane and release the virus genome into the cytosol (Marsh and Helenius, 1980). The viral genome is capped and polyadenylated, so once the virus genome is released into the cytosol, it is treated as cellular mRNA by cellular translational machinery. Translation begins at the 5' end and the nsPs are produced as a single polyprotein. In many alphaviruses, including SINV, that an opal termination codon exists between nsp3 and nsp4. This produces a translational preference for an nsP123 polyprotein rather than the longer nsP1234 polyprotein. Readthrough can only occur at a low frequency (10-20 %). This is one mechanism which small simple viruses use for regulating the translation of specific genes, in this case the RNA-dependent RNA polymerase (nsP4). However, in some alphaviruses, like SFV, this opal codon is generally replaced by a codon for arginine, except in the SFV A7(74) strain. It is suggested that the opal codon may be responsible for the attenuation of SFV A7(74) as the replacement of the opal codon with arginine increases the virulence of SFV A7(74) (Tuittila et al., 2000). This results in a higher proportion of the nsP1234 polyprotein being made (Takkinen, 1986) (Hardy and Strauss, 1988). The polyprotein is cleaved into its individual peptides by the protease nsp2 (Merits et al., 2001). This processing reaction always

occurs in the same order and there is a lag period between each cleavage event. The viral polymerase nsP4 is processed almost immediately, followed by nsP1, nsP2 and nsP3 separately (Kim et al., 2004).

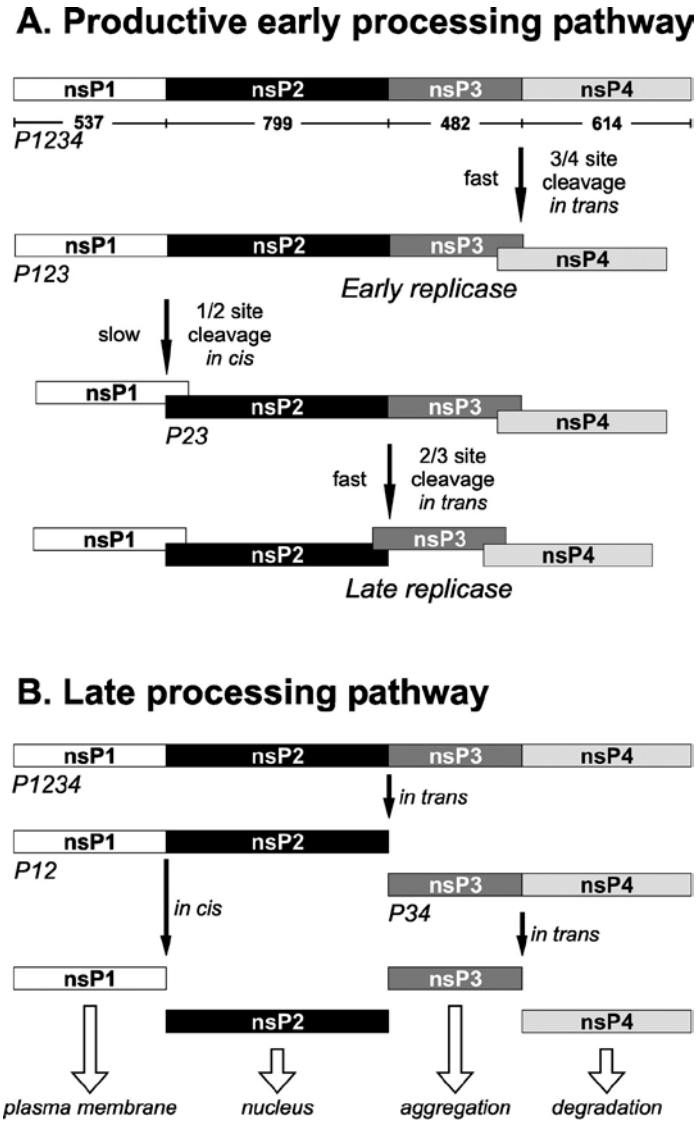

Fig.1.5. Early and late processing of the SFV non-structural polyprotein. (A) Early in infection the polyprotein is associated and produces new genomes. (B) Late in infection, the polyprotein is disassociated and transcripts from the subgenomic promoter are favoured. From (Vasiljeva et al., 2003).

Once the non-structural proteins have been processed, they stay together forming a replication complex that can make copies of the viral genome. Interestingly, timing of the processing of the polyprotein dictates which ORF transcription that takes place. A complex of the nsP123 polyprotein and cleaved nsP4 efficiently produce a negative strand RNA antigenome using the whole positive strand genome as a template. Once nsP1 is processed

and cleaved from the polyprotein, and especially after nsP2 and nsP3 are processed, the complex loses its affinity for making negative strand genomes and concentrates on making positive strand RNA genomes using the antigenomes as a template. Transcription of the positive strand RNA can start at the 3' end so that the whole strand is transcribed or alternatively it can start at the subgenomic promoter located upstream of the structural genes. The subgenomic RNA can be translated separately to make the structural proteins (Kaariainen et al., 1987; Kim et al., 2004).

Each of the nsPs is multifunctional and they are all crucial to virus genome replication, nsP1 is known to have methyl and guanyl-transferase activity, and it caps newly made viral genomic RNAs (Mi et al., 1989; Scheidel & Stollar, 1991). This protein also coats the interior of vesicles, localising the virus genome (to which it is attached) to these cytoplasmic vacuoles that hold alphaviruses replication complexes during infection (Kujala et al., 2001; Spuul et al., 2007) At the cytoplasmic vacuoles, the membranes invaginate inwards to form spherules, in which the replication of virus RNA takes place (Kujala et al., 2001).

The protease nsP2, as mentioned before, is responsible for the processing of the virus polyprotein. However, nsP2 is known to have other functions including NTPase and helicase activity (Rikkonen, Peranen, and Kaariainen, 1994). The nsP1 protein needs the association of nsP2 for the capping of newly made RNA, through its RNA triphosphatase activity (Merits et al., 2001). More importantly, nsP2 has been shown to be an antagonist of cellular interferon (IFN) production (Breakwell et al., 2007) and has also been shown to be cytotoxic when expressed individually from an expression vector. Studies in SINV attribute the cytotoxic effect of nsP2 to its ability to cause a shutdown of transcription, however, this mechanism remains unknown (Garmashova et al., 2006). Mutations in SFV nsP2 can reduce virus cytotoxicity, although this can often be due to a reduction in RNA synthesis and viral replication rather than a direct cytotoxic effect of nsP2.

nsP3 is a phosphoprotein but its function is as yet unclear. Although some research suggests it plays a role in minus strand synthesis (De et al., 2003; Peranen et al., 1988; Wang et al., 1994) other studies suggest it has a role in creating the viral replication vesicles in the cellular membranes (Fros et al., 2012). nsP3 has also been associated with the mammalian protein G3BP and the mosquito homologue, Rasputin. Both of these factors are essential for the assembly of stress granules (Fros et al., 2012). As mentioned before, nsP4 is the RNA dependent RNA polymerase that is essential for virus genome replication. The nsP4 protein

has also been shown to have protease activity as it contributes to its own cleavage from nsP3 (Kaariainen et al., 1987).

Once the subgenomic RNA is transcribed, translation of the structural proteins in this ORF begins. As with the non-structural proteins, the structural proteins are translated as a polyprotein. The capsid protein has self-peptidase activity and cleaves itself from the polyprotein very quickly (Kaariainen et al., 1987). The p62 protein and the E1 glycoprotein are preceded by a signal sequence and a transmembrane domain, which directs the synthesis of the following polyprotein to the ER (Bonatti et al., 1984; Melancon and Garoff, 1986). The ER and golgi then process the proteins and traffic them to the cell membrane. The processed glycoproteins then embed into the cell membrane while the capsid proteins assemble. The capsid proteins interact with a packaging signal present on the virus RNA genome and this initiates assembly of the nucleocapsid core (Geigenmuller-Gnirke et al., 1991). The nucleocapsid then interacts with the cytoplasmic tail of the E2 protein and which induces budding of the virus particle (Lopez et al., 1994). The 6K protein is important for the correct assembly of budding particles and if absent, virus particles are still made, however, they have incorrect glycoprotein spike-like structures and reduced infectivity (McInerney et al., 2004).

SFV

Semliki Forest virus (SFV) is an alphavirus occurring in sub-Saharan Africa and it was originally isolated from *Aedes abnormalis* mosquitoes in 1942 in the Semliki Forest in Uganda, hence its name (Smithburn, Haddow, and Mahaffy, 1946). SFV is mainly transmitted by two species of Aedes mosquitoes, *Aedes africanus* and *Aedes aegypti* (Mathiot et al., 1990). Natural hosts included monkeys and small mammals, however, horses and humans have been reported to be dead end hosts. In human this virus causes a mild febrile illness with symptoms including fever, myalgia, arthralgia and persistent headaches. Only one lethal SFV infection has been documented in humans; a laboratory worker in Germany, who was probably immunocompromised, was infected with a strain of SFV that has now been removed from laboratory circulation (Willems et al., 1979). In 1987, SFV was isolated from blood samples of 22 French soldiers with symptoms of a mild febrile illness (Mathiot et al., 1990). There are several strains of this virus that infect experimental animals including; mice, rats, rabbits and guinea pigs (Bradish, Allner, and Maber, 1971). Originally, two cell lines were found to be permissive for SFV infection and they were commonly used for *in vitro* studies (Atkins, Sheahan, and Mooney, 1990), chicken embryo fibroblasts (CEFs) and baby hamster kidney (BHK-21) cells. However, this virus has the ability to infect many other cell types, from Vero cells to mosquito and tick cells (CJ Leake, 1980; Peleg, 1971). SFV infection of laboratory mice is a useful model for the study of viral induced pathogenesis and encephalitis (Fazakerley, 2004). There are five strains of SFV which have been studied in detail in mammals. The strains known as L10 and prototype are virulent in adult mice (Seamer, Randles, and Fitzgeorge, 1967) (Bradish, Allner, and Maber, 1971). A7, A7(74) and SFV4 strains are avirulent in adult mice and virulent in neonatal mice (Pusztai, Gould, and Smith, 1971) (Atkins, 1983). Age dependent death for mice with avirulent SFV strain is unclear. It has been suggested that susceptibility of neurons to undergo apoptosis in response to SFV infection might play a role in this process (Fazakerley, 2002). Multiple sequence differences have been found between virulent and avirulent strains. These differences occur in the nsP2 and nsP3 genes of the replicase as well as in the E2 glycoprotein which have been implicated in mammalian neuropathogenicity (Fazakerley, 2002; Santagati et al., 1995; Santagati et al., 1998; Tuittila et al., 2000). So far only two strains of SFV have been investigated in mosquito cell culture systems, SFV4 and SFV-A7(74).

SFV infection of mosquito cell culture is characterised by a viremic acute phase, followed by a decline and persistent phase (Siu et al., 2011b). During SFV infection of *Ae. albopictus*

derived U4.4 cells, the number of cells in the culture does not vary from an uninfected culture. The decline in virus production after the acute phase is possibly due to the cellular innate immune response, in particular RNAi, although SFV is known to interact and even inhibit some of the other cellular immune signalling pathways (Fragkoudis et al., 2008c). The specifics of how SFV interacts with the mosquito cell innate immune system will be discussed later in this chapter. SFV has proven a very valuable laboratory arbovirus model for investigating innate immune responses against arboviruses (Fragkoudis et al., 2008c; Siu et al., 2011b).

SFV as a model and a tool

The genome of SFV is a typical alphavirus genome; it contains only 9 genes and has a total size of 11.5 Kb. Alphaviruses are often used as viral vectors to express foreign genes in cells. SFV vectors have been developed as vaccine delivery systems and for therapeutic gene therapy (Atkins, Sheahan, and Liljestrom, 1996; Hoffmann et al., 2001; Lundstrom, 2003). Infectious DNA clones can be created and manipulated using SFV complementary deoxyribonucleic acid (cDNA). Foreign genes can be inserted into specific locations and then expressed efficiently upon infection of mammalian or mosquito cells. As will be described in Chapter 5, the SFV genome can be engineered with a ZsGreen attached to nsP3 or a *Renilla* luciferase (R*Luc*) gene cleaved from the same protein by a cellular peptidase. More excitingly, a second subgenomic promoter could be added to the SFV genome to express a foreign gene in large amounts (Tamberg et al., 2007).

Biosafety of recombinant SFV systems can be increased by using replication deficient vectors which only undergo one round of infection. SFV replicons are structurally identical to normal SFV but the encapsidated genome lacks sequences for the structural genes. A foreign gene sequence can be substituted in its place for example, a sequence coding for a short half-life, enhanced green fluorescent protein (d1eGFP). The inserted foreign gene (d1eGFP) is under the control of the virus subgenomic promoter and is expressed when the structural proteins would normally be expressed (Smerdou & Liljestrom, 1999).

SFV is closely related to CHIKV, however, it lacks the pathogenicity of this virus. Therefore, SFV can be used as a model in a lower biosafety level.

Arthropod vectors

As mentioned in this introduction, many arthropods are potential or proven arboviral vectors, this includes mosquitoes, ticks, midges and sandflies. In this study, *in vitro* experiments were carried out with *Ae. albopictus* derived cells whereas *in vivo* experiments were done with *Ae. aegypti* Liverpool red eye strain mosquitoes optimised for filarial growth, that were kindly provided by R. M. Maizels and Y. Harcus (Institute of Immunology and Infection Research, University of Edinburgh).. In the following section the intricate aspects of the *Ae, albopictus* ecology and interactions with the virus will be discussed.

Ae. albopictus: classification, geographical distribution, life cycle and disease transmission

The common name of *Ae. albopictus* is the Asian tiger mosquito. This name could refer to the spotty pattern (albopictus = white painted) or also to its high level of aggression (Skuse, 1894). Although it originated in tropical Asia, it can now be found in Europe, the Americas, Africa and a number of locations in the Pacific and Indian Oceans (Paupy et al., 2009). *A. albopictus* geographical spread has occurred in the last three decades, following the spread of *Ae. aegypti* and previously *Culex pipiens*. All of these cases are examples of competent mosquito vectors spreading due to human action (Lounibos, 2002). Initially, the spread of *Ae. albopictus* was considered a reminder of the impact of globalization and a minor public health threat. Ironically, *Ae. albopictus* was also considered as a potential way to control *Ae. aegypti*, because of the competition between both species. However, its vector status drastically changed due to the CHKV epidemic during 2006-2007 in the Indian Ocean, Africa and Italy, when it became the interest of extensive of research (Mavalankar et al., 2008; Nandha and Krishnamoorthy, 2009; Robin et al., 2008).

In the forest of Southeast Asia where it originated, *A. albopictus* was most probably zoophilic. However, it is likely that it slowly became anthropophilic due to changes in habitat and the availability of new blood sources such as domestic animals and humans. This process is known as domestication, and it has been also reported for *Ae. aegypti* in Africa (Tabachnick, 1991). These days, *Ae. albopictus* dwells in rural and suburban areas of Asian cities, such as Kuala Lumpur, Singapore and Tokyo (Paupy et al., 2009). Spread from Southeast Asia was facilitated by intercontinental travel. It has been suggested that *Ae. albopictus* was introduced into the USA in a shipment of tires in 1985 (Hawley et al., 1987;

Reiter and Sprenger, 1987). Since then *Ae. albopictus* has colonized most of the Americas, from USA to Argentina, the Pacific Islands and Australia. In 1989 it made its way to South Africa and then later to Nigeria, Cameroon, Equatorial Guinea and Gabon. In Europe it was first detected in Albania in 1979 and later it extended to the rest of the Balkan peninsula, Italy, France, Switzerland, Netherlands and Spain (Scholte, 2007).

Ae. albopictus success is due to its physiological and ecological plasticity. It can survive colder northern climates much better than *Ae. aegypti* (Mitchell, 1995). A comparison between *Ae. albopictus and Ae. aegypti* vectors will be discussed later. *Ae. albopictus* is also very adapted to live in a range of environments, from tropical jungles, to dense urban areas (Paupy et al., 2009). It is a daytime biter and has a wide range of hosts, from reptiles and amphibians, to birds and mammals (Scholte, 2007). However, unlike *Culex pipiens*, it prefers to feed on humans in urban areas rather than on birds or other warm-blooded animals (Munnoz et al., 2011). Due to the wide range of habitat, *Ae. albopictus* was initially suggested as a 'bridge vector' for the transmission of emerging viruses between animals and humans, and *Ae. aegypti* would maintain these viruses in the urban cycle. However, it is now understood that *Ae. albopictus* can also keep urban cycles of arboviral diseases (Delatte et al., 2008; Paupy et al., 2009).

Fig.1.6. Aedes albopictus at different life stages. Adult female taking a blood meal from a human (left) Third and fourth instar larvae (right)(www.vectorbase.org) (Paupy et al., 2009).

The ability of *Ae. albopictus* to act a vector has been experimentally proven for 26 viruses belonging to the *Bunyaviridae*, *Flaviviridae*, *Reoviridae* and *Togaviridae* families (Gratz, 2004; Moore and Mitchell, 1997). However, only 14 viruses have been isolated in the wild from these mosquitoes: six flaviviruses (DENV1-4, WNV, JEV), two alphaviruses (CHIKV and EEEV) and six bunyaviruses (Potosi virus, Tensaw virus, Keystone virus, La Crosse virus and Jamestown Canyon virus). However, to the date, *Ae. albopictus* only plays a significant role in the transmission of DENV and CHIKV (Paupy et al., 2009). Even if *Ae. albopictus* can cause epidemics of DENV, *Ae. aegypti* is the major vector. However, it is clearly the CHIKV outbreaks of the last decade where *Ae. albopictus* becomes of interest as the main vector of the disease (Angelini et al., 2007a; Leroy et al., 2009; Pastorino et al., 2004). Vector competence was achieved by just a single adaptive mutation in the E1 gene (a valine residue replacing alanine at position 226) probably by sporadic contact between the virus and *Ae. albopictus* (de Lamballerie et al., 2008). This mutation provided a selective advantage for the transmission, replication and vector competence of the new CHIKV strain by *Ae. albopictus* (Vazeille et al., 2007). This enabled the virus to expand its geographical range across the Indian Ocean to other places where *Ae. albopictus* was present. The repercussions of the CHIKV endemic area expansion were discussed in the alphavirus section of this chapter.

The spread of *Ae. albopictus* transmitted diseases around the globe is affected by several factors like climate, environment, globalization and mosquito control (Paupy et al., 2009). It is expected that *Ae. albopictus* transmitted disease will expand, especially in Central America and sub-Saharan Africa, since there are a high number of potential niches for this species and a number of transmissible diseases that are endemic in those areas, such as YFV (Benedict et al., 2007). Climate can be considered a secondary factor for the spread of *Ae. albopictus*, even in the presence of climate change. This mosquito is adapted to extremely variable temperature ranges, although, colder temperatures have been correlated with lower arboviral transmission rates in *Ae. albopictus* as vertical transmission reduces vector and viral fitness (Lambrechts and Scott, 2009). Historically, YFV and DENV infections occurred further north than in recent times, like Brest in France, Glasgow in Scotland and Boston in the USA (Barrett, 2000). Environmentally *Ae. albopictus* is very adaptable and in larval stages it outcompetes other *Aedes* species. As adults they adapt their feeding to almost any animal available in that area (Delatte et al., 2008). Globalization is perhaps the factor that predominately affects the spread of *Ae. albopictus*, as was mentioned before, due to the

transport of merchandise (tires in the US or potted plants in Holland's greenhouses)(Hawley et al., 1987; Reiter and Sprenger, 1987; Scholte, 2007).

It can be concluded that geographical expansion of *Ae. albopictus* will increase in the next coming years. Moreover, even in the temperate areas where this vector has been established, the R_0 transmission value is known to be rising near to one, creating the opportunity of human to human transmission (Angelini et al., 2007b).

Interactions between aedine mosquitoes

It is interesting to notice that in areas where both *Ae. aegypti* and *Ae. albopictus* exist, both species share the same larval rearing niches. In Central Africa and the Americas there are some urban and suburban habitats where both species co-occur (Braks et al., 2004; Juliano, Lounibos, and O'Meara, 2004; Simard et al., 2005). However, there is a trend that will eventually result in the disappearance of *Ae. aegypti* from areas where these two species of mosquitoes co-exist (Bagny et al., 2009a; Bagny et al., 2009b). This displacement of the endemic *Aedes* species has been observed in many countries after the introduction of *Ae. albopictus*. The most likely reason is the competition for the available food among the larvae (Paupy et al., 2009). Experiments addressing this hypothesis have been done both in laboratory conditions and in the field, however, the question has remained inconclusive, most probably due to the experimental conditions and the use of laboratory mosquito strains for these studies. The use of artificial food in laboratory experiments has demonstrated a significant advantage of *Ae. aegypti* in the USA and Asia, where this species is an invader (Barrera, 1996; Moore and Mitchell, 1997). However, other similar field studies taking place in the USA have suggested the opposite, that *Ae. albopictus* is favoured over *Ae. aegypti* due to larval competition over limited food sources (Juliano, Lounibos, and O'Meara, 2004). It has been suggested that *Ae. albopictus* is responsible for the decline of local Brazilian populations of *Ae. aegypti* twenty years after this mosquito was introduced to the country, which was confirmed by field and laboratory container studies in this country (Braks et al., 2004).

One interesting aspect that should be highlighted is that larval competition between both species increases arbovirus transmission. Studies suggest that for *Ae. albopictus* mosquitoes that competed at larval stages with *Ae. aegypti* have higher infection rates and SINV titres than those that did not (Alto et al., 2005). The same was reported with DENV in a similar

study (Alto et al., 2008). The difference was only significant in *Ae. albopictus*, and species competition played no role in the increase of arbovirus transmission in *Ae. aegypti* mosquitoes. Vector competence enhanced by species competition is suggested to occur as a result of a physiological barrier reduction and could contribute to the efficiency of *Ae. albopictus* in transmitting DENV.

Ironically, it has been suggested that *Ae. albopictus* could be introduced to control of *Ae. aegypti* populations due to its lower vector competence. Events in the past decade proved this statement wrong and luckily the idea was not put in practice. Understanding the mechanisms of vector competence as well as the molecular relations and adaptations between the virus and the mosquito are key when formulating strategies for controlling arboviral infections.

Mosquito life cycle

There are four stages to the mosquito life cycle: egg, larva, pupa and adult. Female *Ae. aegypti* become fecundated before taking a blood meal and start initiating the process of egg production. However, this process is arrested at the vitellogenesis step (synthesis of yolk protein precursors) until the female has found a host and taken protein rich blood. Taking a blood meal involves drastic changes in the female mosquito physiology. A large number of genes are induced which include digestive enzymes, as well as the creation of a series of membranes around the midgut. Gene induction also lifts the repression on yolk protein precursor genes (YPPs) once blood feeding has taken place (Attardo et al., 2003). Male mosquitoes only feed on sugar rich plant nectar and do not require a blood meal. A female mosquito can lay several hundred fertilised eggs on the surface of water in tree holes, or other water-holding containers. Eggs can be clustered or dispersed depending on the species of the mosquito. In some mosquitoes eggs can survive desiccation and other extreme weather conditions, hatching only when the environment is appropriate. Mosquito larvae can hatch from the eggs as soon as within 24 hours. The larvae feed on plant material, algae, bacteria, and other micro-organisms. Domesticated larvae kept in an insectary can be fed on commercial chicken liver powder or yeast extract. The larvae take 7-14 days before moulting into pupae. Mosquito pupae spend 1-4 days transforming into adult mosquitoes (metamorphosis) and then leave the pupal case.

Virus infection and dissemination in mosquitoes

Arboviruses have to undergo replication in the mosquito vector in order to be efficiently transmitted between vertebrate hosts. The virus has to pass several physical and immunological barriers between the blood meal and secretion from the salivary glands. Initially, arboviruses are ingested by the mosquito through a blood meal from a viraemic host. The viral titres of this infectious blood meal vary between hosts and viruses but it is usually considerably high, with an average of 10^7 PFU per ml of blood (Weaver, 1997). Viral titre, as well as number of feeding sessions, increases the virus infection rate of the vector. Once the viral infected blood meal has entered the mosquito, it is digested in the midgut. The arbovirus has to be able to infect the midgut cells. This step is restricted by the receptors in the midgut cells, as well as by digestive enzymes which process the virus glycoproteins (Tchankouo-Nguetcheu et al., 2010). The mosquito midgut is the major barrier for pathogen transmission. After entering the midgut cells, sufficient replication must then occur for the virus to enter the heamocoel (Woodring JL, 1996). Between the midgut and the haemocoel there are two different tissues known as the matrix and the basal lamina that the progeny virus has to cross. Midgut basal lamina is a porous cell barrier impermeable to arboviruses (Romoser et al., 2005). The matrix is a temporary membrane that the mosquito creates around the midgut after taking a blood meal and it is also impermeable to arboviruses (Kato et al., 2008). It is suggested that arboviruses, when infecting these tissues, can change cell morphology and attachment, inducing motility of the cells thus disrupting and permeabilising these barriers by the use of matrix metalloproteinases (Wang et al., 2008b). Arboviruses can then infect other tissues such as the fat body and the muscle surrounding the alimentary tract and be distributed through the body of the vector by the haemolymph (infection of haemocytes) or by infecting tracheal tissue (Woodring JL, 1996). Finally, the virus reaches the salivary glands where it replicates at a higher titre than it does in other tissues (Arcà et al., 2007; Valenzuela et al., 2002). The reason for these higher salivary gland viral titres is still unknown. Viruses are then exported to the saliva and they can be transmitted to another vertebrate host following a bite and a bloodmeal.

One interesting aspect of the arbovirus cycle in mosquitoes is the suggestion of a change of behaviour in the successfully infected vector. Mosquitoes infected with several arboviruses have been reported to display increased motility, host-seeking behaviour even in the presence of repellents (DEET) and number of feeding sessions (Lima-Camara et al., 2011; Qualls et al., 2012a; Qualls et al., 2012b). Transcriptomic studies of DENV infected *Ae. aegypti*

females show an unusual upregulation of several olfactory proteins, which could be one of the mechanisms explaining how a virus can change vector behaviour (Das et al., 2010; Sim, Ramirez, and Dimopoulos, 2012).

Mosquito cell lines

In order to understand the most basic molecular mechanisms of arbovirus infection of mosquitoes several *Aedes* derived cell lines have been used. These cell lines are valuable tools and they were mainly derived from mosquito larvae. Three cell *Ae. albopictus* will be discussed as they are the most popular ones used in arbovirus studies. The origin of the *Ae. albopictus* and *Ae. aegypti* derived cell lines can be traced to common ancestral obscure cultures known as 'Singh' cells, the name of the scientist who started characterising them. These cell lines were formed by heterogeneous population of cell types cultivated from ground mosquito larvae. These cells were selected for arbovirus growth (Paul, Singh, and Bhat, 1969; Singh and Paul, 1968; Singh and Paul, 1969) and their culture requirements were described. Cell cultures were also distributed and, occasionally, cloned (named C3, C6, C7, etc) (Bhat and Singh, 1971; Singh, 1971).

The most widely used *Ae. albopictus* derived cells are known as C6/36 (Igarashi, 1978). These cells were isolated from Singh cell lines from two different C6 cultures (SAAR from the USA and SAAK from Kobe, Japan). These two cultures were combined, propagated and selected in several stages for the growth of JEV and SINV (Igarashi, Sasao, and Fukai, 1973) (Stollar et al., 1975). In the final stages, clones were isolated in the presence of CHIKV serum (Igarashi, 1978). They were tested and selected for their yield of DENV (for all its serotypes) and CHIKV. Interestingly, the original clone of C6/36 showed, together with a much higher yield of DENV and CHIKV, cytopathic effects following infection by these viruses, in contrast to the original clone of (SAAR) cells. However, the cells later became persistently infected by these viruses and cytopathology was greatly reduced (Igarashi, 1979). Currently, these cells are used to grow arboviruses due to their high production yield. However, it has been recently described that these cells have a dysfunctional RNAi pathway due to a truncation in the key protein Dicer-2, hence the higher viral yield (Brackney et al., 2010). In these cells however, stimulation of the Imd pathway seems to have an antiviral effect (Avadhanula et al., 2009; Fragkoudis et al., 2008b). The role of the innate immunity pathways against arboviruses in mosquitoes will be described later in this chapter.

Another widely used *Ae. albopictus* derived cell line is known as C7-10. These cells also originated from Singh cell lines. They were selected as being temperature sensitive and produce higher yields of JEV, VSV and SINV (Sarver and Stollar, 1977). These cells were subcloned from the C7 culture known as LT C-7 and, as occurred with C6/36, the cytopathic effects of arboviruses in these cell lines were eventually eliminated. Interestingly, also like in C6/36, the Dicer-2 protein of the antiviral RNAi in this cell line also seems to be defective due to a deletion, although not as extensively as in C6/36 (Brackney et al., 2010).

The final widely used *Ae. albopictus* cell line, and which was used in this study, is U4.4. These cells are considered the direct ancestral descendent of the *Ae. albopictus* derived Singh cells, without suffering any subcloning (Bhat and Singh, 1971; Gliedman, Smith, and Brown, 1975; Miller and Brown, 1992) These cells share the ancestral culture heterogenicity of cells with different morphology. U4.4 cells have also shown a reduction of virus release overtime when infected with SINV, unlike C6/36 and C7-10 cells. Millter and Brown also observed that, even with reduced viral production, most of the cells remained persistently infected in the culture. These cells were used for the study in this thesis since they have intact innate immunity signalling pathways (Fragkoudis et al., 2008c).

As was mentioned before, the initial arboviral infection of mosquito cell culture starts with an acute phase and a high titre of virus produced. After 16 hours the virus enters a persistent phase with a reduction in titre. Bringing the virus infection from an acute to a persistent phase is mediated by the cellular innate immunity, which can strongly reduce viral replication and production without completely clearing the pathogen from the cell.

Innate immunity in mammals

In vertebrates IFNs are the main player in controlling a viral infection and are a group of cytokines secreted by vertebrate cells. Type I IFN, IFNα and IFNβs are mainly produced by leukocytes and fibroblasts (Isaacs and Lindenmann, 1957). This production is triggered by pathogen recognition receptors (PRRs) which detect conserved foreign molecular motifs, Pathogen-associated molecular patterns (PAMPS), including surface glycoproteins, ssRNA, dsRNA and unmethylated CpG DNA-islands. There are two types of PRRs that are known to recognize viral PAMPS: Toll-like receptors (TLRs) which reside in the plasma membrane or the endosomal compartments and retinoic acid-inducible gene I (RIG-I)-like receptors, which reside in the cytoplasm (Alexopoulou et al., 2001; Foy et al., 2005).

The TLR family is formed by up to 11 transmembrane proteins, of which six of them (TLR2, TLR3, TLR4, TLR7, TLR8 and TLR9) are known to have a role in antiviral immunity (Galiana-Arnoux and Imler, 2006). TLR2 and TLR4 can be activated by viral surface glycoproteins (Galiana-Arnoux and Imler, 2006); TLR7 and TLR8 are triggered by ssRNA (Wang et al., 2005b); TLR3 is engaged by extracellular dsRNA112; and TLR9 is activated by unmethylated CpG-containing DNA (Miller, Meng, and Tomai, 2008).

TLRs include RNA helicases (such as MDA5 (melanoma differentiation-associated protein 5; also known as IFIH1), RIG-I and PKRs (dsRNA-dependent protein kinases) detect viral RNA in the cytoplasm (Baum and Garcia-Sastre, 2010). Since most arboviruses are ssRNA viruses that replicate with a dsRNA intermediate, potential sensors include TLR3, TLR7, TLR8 and the RLRs.

Expression of antiviral gene products can trigger dramatic changes in the cell, such as the induction of mRNA degradation, translational shut-down and, in many cases apoptosis and death of the infected cell. Therefore, the INF response must be tightly controlled to avoid severe host damage (Randall and Goodbourn, 2008).

Innate immunity in arthropods/insects

Arboviruses have to be able to replicate both in the vertebrate and invertebrate system. The interactions between the mosquito and the arbovirus are not always benign to the vector, although, infection of arthropod cell cultures usually develops into a persistent infection after an acute phase with high virus production. This efficient control of virus replication and production it is thought to be due to the innate immune system of the arthropod. Unlike vertebrates, arthropods do not have an INF response, although they are known to secrete an array of antiviral molecules and are known to have powerful antiviral cellular pathways. This topic has been extensively reviewed in (Fragkoudis et al., 2009), (Merkling and van Rij, 2012) and (Blair, 2011).

The Toll cascade

Insects rely on the Toll pathway in order to mount an immune response against fungal pathogens and Gram-negative bacteria. Most of the advances in understanding this pathway have been carried out in drosophila, however, although it works in a similar way in mosquitoes, slight differences arise in the haemotophagus vectors that will be pointed out later. In drosophila, the Toll pathway shares similarities with vertebrate Toll-like receptor signalling (to whom gave its name) and Interleukin-1 (Lemaitre and Hoffmann, 2007).

The Toll pathway in drosophila is triggered by Gram-positive bacteria and fungi through two different sets of receptors. The first set recognises PAMPs, such as fungal beta-glucans and Gram-positive lysine-type peptidoglycans by PRRs, in this case Gram-negative binding protein (GNBP)-1 and -3 for beta-glucans, and peptidoglycan receptor proteins (PGRP)-SA and PGRP-SD for Gram-positive bacteria cell walls (Gottar et al., 2002; Tauszig et al., 2000). The second receptor is the haemolymph protease Persephone, which can sense the proteolytic activity that occurs during microbial infection of the haemolymph and triggers a danger signal (Chamy et al., 2008; Gottar et al., 2006). Both sets of signalling cascades occur extracellularly and lead to the proteolytic cleavage of pro-Spatzle into the active signalling cytokine Spatzle, which subsequently binds to the transmembrane receptor Toll. An intracellular signalling cascade then allows the translocation of NF-κB like transcription factors to the nucleus. These factors are known as Dif (Dorsal-related immunity factor) which is present in adult flies, and Dorsal, present both in larvae and adults. Once in the nucleus, Dif and Dorsal bind to NF-κB transcriptional factors and they induce the transcription of

several genes, among them a specific set of antimicrobial peptides (AMP), such as drosomycin and *Defensin* (Fig.1.7) (Lemaitre and Hoffmann, 2007).

In mosquitoes, the annotation of *A. gambiae, C. quinquefasciatus* and *Ae. aegypti* genomes has thrown some light onto the mechanisms of the Toll pathway in these insects (Bartholomay et al., 2010; Christophides et al., 2002; Holt et al., 2002; Nene et al., 2007; Waterhouse et al., 2007). It is known that mosquitoes lack Dif and that they solely rely on the Dorsal orthologue Rel1 to induce AMPs through this pathway. *Ae. aegypti* expresses two isoforms of the Rel1 protein, Rel1-A and Rel1-B, which cooperate to enhance gene expression (Shin et al., 2005).

In addition to bacteria and fungus infection this pathway also has an antiviral role. Infection of flies by Drosophila X virus (DXV) induces production of drosomycin at levels comparable to those triggered by *E. coli*. In addition, mutant flies with defective *Dif* were more susceptible and succumbed to viral infection (RA et al., 2005). In DENV infected blood fed mosquitoes, microarray transcriptional profiling identified 240 genes that were up highly induced (Xi, Ramirez, and Dimopoulos, 2008). Among the up-regulated genes there were several components of the Toll pathway, such as Spatzle, Toll, Rel1 and AMP-like defensin. In the same study, when Toll adaptor protein MyD88 is silenced by RNAi, viral titres in the mosquito midgut increased which suggests the importance of this pathway in controlling DENV in *Aedes aegypti* mosquitoes. In WNV infected *C. pipiens* resulted in up-regulatation of an unknown Toll receptor (Smartt et al., 2009). Toll seems to play a role in antiviral immunity against flaviviruses, however, its role in alphavirus infection is unclear. SFV infection of *Ae. albopictus* derived U4.4 cells is not affected by Toll signalling (Fragkoudis et al., 2008c). In addition, another microarray study of SINV infected *Ae. aegypti* mosquitoes only detected a modest 1.8 fold induction of Rel1, and neither Rel1, nor other Toll related genes were up regulated in later time points (Sanders et al., 2005).

The Imd cascade

In drosophila, PGRP-LC and LE detect diaminopimelic (DAP) type peptidoglycans from Gram-negative bacteria and trigger the Imd pathway. The PGRP activated receptors do this by recruiting Imd and triggering an intracellular signalling cascade that proteolytically activates another NF-κB like factor, pro-Relish, into Relish by cleavage of its inhibitory IκB domain (Lemaitre and Hoffmann, 2007). Once activated, the *Rel* domain from Relish

translocates into the nucleus and induces transcriptions of AMPs such as *Cecropin* and *Diptericin*. As occurs with Toll, the end effectors molecules of this pathway are different in mosquitoes. The mosquito orthologue of drosophila Relish is known as Rel2 in *A. gambiae* and *Ae. aegypti* mosquitoes (Christophides et al., 2002; Waterhouse et al., 2007). While only two Rel2 isoforms exist in *A. gambiae*, in *Ae. aegypti* Rel2 occurs in three isoforms (short, long and IκB type) (Meister et al., 2005; Shin et al., 2002). Rel2-long is similar to drosophila's Relish, it is the predominant isoform and contains histidine/glutamine-rich and serine-rich regions, Death domains, REL-homology domains and inhibitor-like IκB-like ankyrin. On the other hand, Rel2-short lacks both the ankyrin and Death domains. The IκB-type consists solely of an IκB domain (Shin et al., 2002).

In drosophila two studies have linked Imd with antiviral immunity (Avadhanula et al., 2009; Costa et al., 2009). The first studies suggests that distinct branches of the Imd pathway contribute differently to antiviral immunity while activation of the Imd pathway and AMP is not related to gene induction in response to cricket paralysis virus infection (CrPV) of the fly (Costa et al., 2009). In the other study, transgenic drosophila expressing a SINV replicon and mutant flies of several components of the Imd pathway such as Relish, Imd, dFADD and Dredd showed higher viral RNA levels. In this study it was also observed that viral replication induces the Imd-dependent AMPs Dipterin and Metchnikowin (Avadhanula et al., 2009). However, since SINV by definition does not leave the cells, thus does not trigger extracellular receptor, these genes could be cross activated by other mechanisms. It has been suggested that there might be undefined receptors detecting intracellular viral RNA and triggering the Imd pathway but no experimental evidence has been provided (Merkling and van Rij, 2012).

In mosquitoes, Imd also has a role in controlling alphavirus infection. *A. gambiae* infected by injection with O'nyong'nyong virus (ONNV) showed upregulation of Imd pathway genes (Waldock, Olson, and Christophides, 2012), however, previous findings by this group in mosquitoes infected with ONNV by blood feeding does not show any upregulation of the Imd pathway, in fact a slight downregulation was observed (Sim et al., 2005). In addition, the same study also suggests that silencing key players in the Imd pathway, such as Rel2 and NF-κB does not have any effect on ONNV titres (Sim et al., 2005). Gene expression profiles vary in these studies due to two different means of infecting mosquitoes with ONNV, however, blood feeding can be considered the most natural and less invasive method. SFV infection of *Ae. albopictus* derived U4.4 and C6/36 cells triggers induction of the Imd pathway,

moreover, the induction of this pathway has a negative effect in viral replication (Fragkoudis et al., 2008c).

The JAK/STAT pathway

This evolutionary conserved pathway was first studied for its role in drosophila development (Arbouzova and Zeidler, 2006; Luo and Dearolf, 2001; Zeidler, Bach, and Perrimon, 2000), however, it has also been linked with antimicrobial immunity (Agaisse and Perrimon, 2004; Agaisse et al., 2003; Buchon et al., 2009; Goto et al., 2010). The pathway is triggered in drosophila by the binding of a cytokine, belonging to the Unpaired (Upd) family to the receptor Domeless. Drosophila encodes only a single Jak kinase and a single Stat transcription factor, which, upon activation, dimerizes and translocates to the cell nucleus driving transcription of genes under promoters with Stat binding sites (Agaisse and Perrimon, 2004). In *A. gambiae* this pathway is known to also be triggered by bacterial infection (Barillas-Mury et al., 1999) and *Ae. albopictus* STAT proteins have been characterized (Lin et al., 2004a).

JAK/STAT is also known to have anti-viral properties. Drosophila C virus infection of the fly is known to trigger the pathway through replication, virus induced cell damage and through the production of cell debris (Dostert et al., 2005; Hedges and Johnson, 2008). Several orthologues of the key molecules of the JAK/STAT pathway (domeless, hop, Stat, PIAS, SOCS) have been identified in drosophila, *A. gambiae* and *Ae. aegypti* by comparative genomic studies (Souza-Neto, Sim, and Dimopoulos, 2009; Waterhouse et al., 2007; Zou et al., 2011). Transcriptomic studies show that DENV infection of *Ae. aegypti* upregulates the receptor Domeless as well as other Jak/Stat related genes up to 10 days after an infectious blood meal (Souza-Neto, Sim, and Dimopoulos, 2009; Xi, Ramirez, and Dimopoulos, 2008). Silencing of key genes of the pathway, such as *hop*, resulted in higher DENV titres in the midgut (Souza-Neto, Sim, and Dimopoulos, 2009).

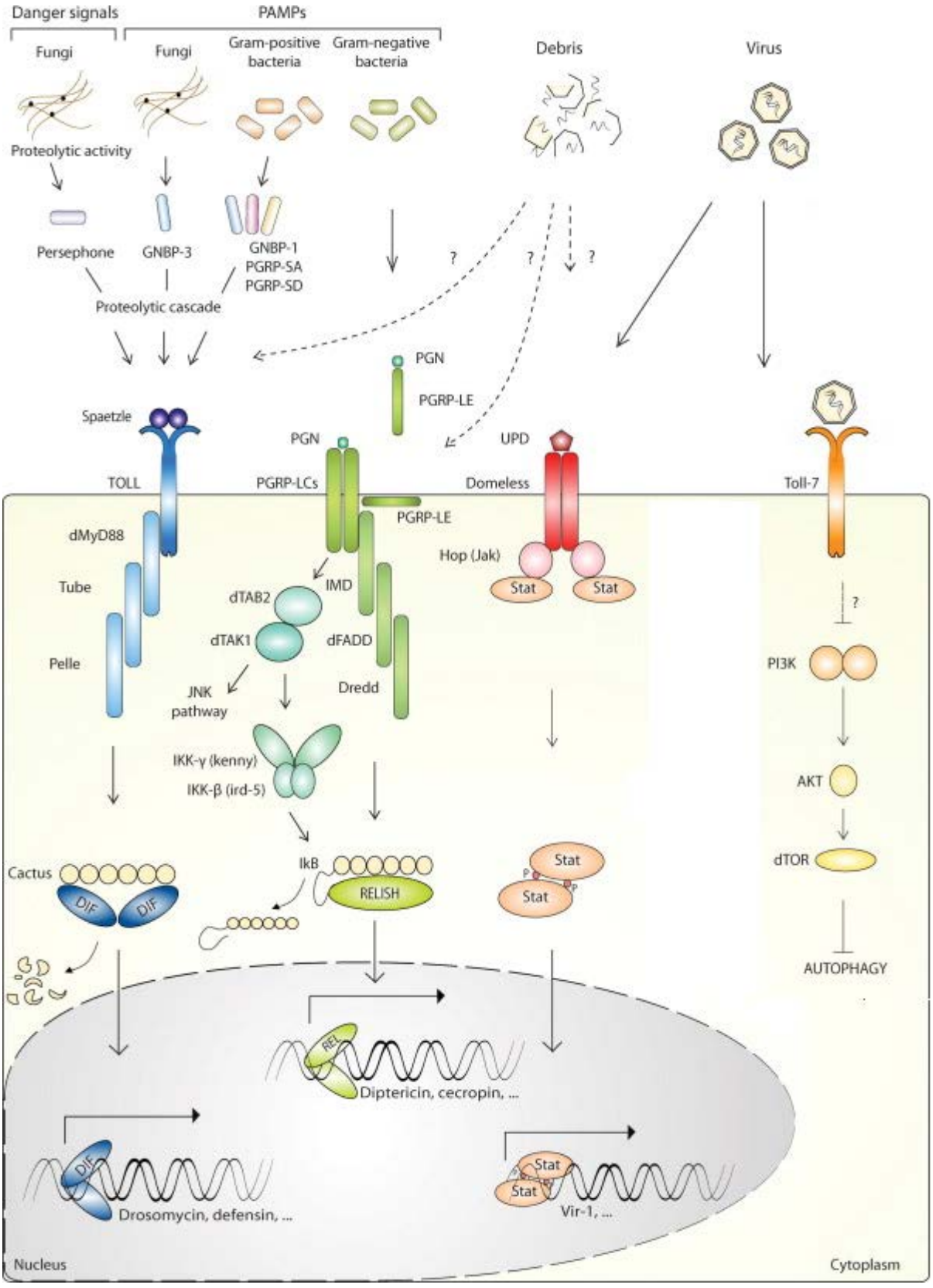

Fig.1.7. Antimicrobial signalling pathways in drosophila. Based on (Merkling and van Rij, 2012).

RNAi

RNA interference (RNAi) is the most important antiviral pathway in insects (Blair, 2011). The insect antiviral immunity response has been characterised recently and it mainly consists of the exogenous RNAi (exo-RNAi). RNAi was first observed in plants in the early 1990s as a defence mechanism against transcriptional errors and it was named post-transcriptional gene silencing or virus induced gene silencing when directed against virus infection (Lindbo et al., 1993). It is now understood that both gene silencing processes belong to the same pathway, RNAi. The molecular trigger for the RNAi pathway is dsRNA as was revealed in studies in *Caenorhabditis elegants* and *Drosophila melanogaster* (Fire et al., 1998; Kennerdell and Carthew, 1998). Most of the knowledge we have about RNAi in insects has been discovered in drosophila and cultured cells. RNAi is, as it was mentioned before, generally the most important antiviral mechanism in insects. In order to mount a response it does not need transcriptional induction and it is still unclear if its activation induces transcription of antiviral genes (Campbell et al., 2008; Deddouche et al., 2008; Dostert et al., 2005; Khoo et al., 2010). RNAi is triggered by intracellular detection of dsRNA by Dicer-2 (Dcr-2). This molecule is an RNase III endonuclease that acts as a PAMP, recognising cytoplasmic dsRNA and cleaving it into small interfering RNAs (siRNAs) as a first step in the initiation of the RNAi pathway (Bernstein et al., 2001). Dcr-2 cleaved siRNAs are usually 21 bp in length with 5' phosphates and two nucleotide overhangs on the 3' hydroxyl end (Elbashir et al., 2001; Siu et al., 2011b) . The siRNAs, together with Dcr-2 and R2D2 (an dsRNA binding proteins) are loaded into the RNA-induced silencing complex (RISC), which contains the key molecule Argonaute-2 (Ago2) in addition to probably many more, presumably uncharacterised proteins (Liu et al., 2003; Okamura et al., 2004). In the RISC complex one of the siRNA strands is unwound and degraded and the guide strand is used as a guide for recognition and annealing of complementary ssRNA sequences. Once a complementary sequence has been found and targeted, Ago-2 cleaves this transcript (Miyoshi et al., 2005; Schwarz et al., 2002; Schwarz, Tomari, and Zamore, 2004). RNAi drastically inhibits viral replication without damaging the host cell.

In mosquitoes RNAi works in a similar way to that in the previously described drosophila system. Antiviral RNAi in these vectors has been reviewed in great detail (Blair, 2011). After the publication of the complete mosquito genomes of *A. gambiae*, *Ae. aegypti* and *C. quinquefasciatus* the identification of orthologs of the drosophila RNAi pathway, such as Dcr-2, R2D2 and Ago-2 was possible (Arensburger et al., 2010; Holt et al., 2002; Nene et al.,

2007; Waterhouse et al., 2007). Viral replication intermediates provide the dsRNA necessary for triggering the RNAi pathway. Silencing of any of the proteins in the RNAi pathway resulted in an increase in virus replication *in vitro* and *in vivo* (Campbell et al., 2008; Sanchez-Vargas et al., 2009). siRNAs of viral origin are known as viRNAs (viral induced siRNAs), they are derived both the genome and antigenome and are distributed along the whole length of the genome in flaviviruses and alphavirus infection (Scott et al., 2010; Siu et al., 2011b). Furthermore, it has been shown that, as occurs in drosophila, mosquito cells can spread viRNAs from cell to cell in order to ready them for an antiviral response (Attarzadeh-Yazdi et al., 2009)

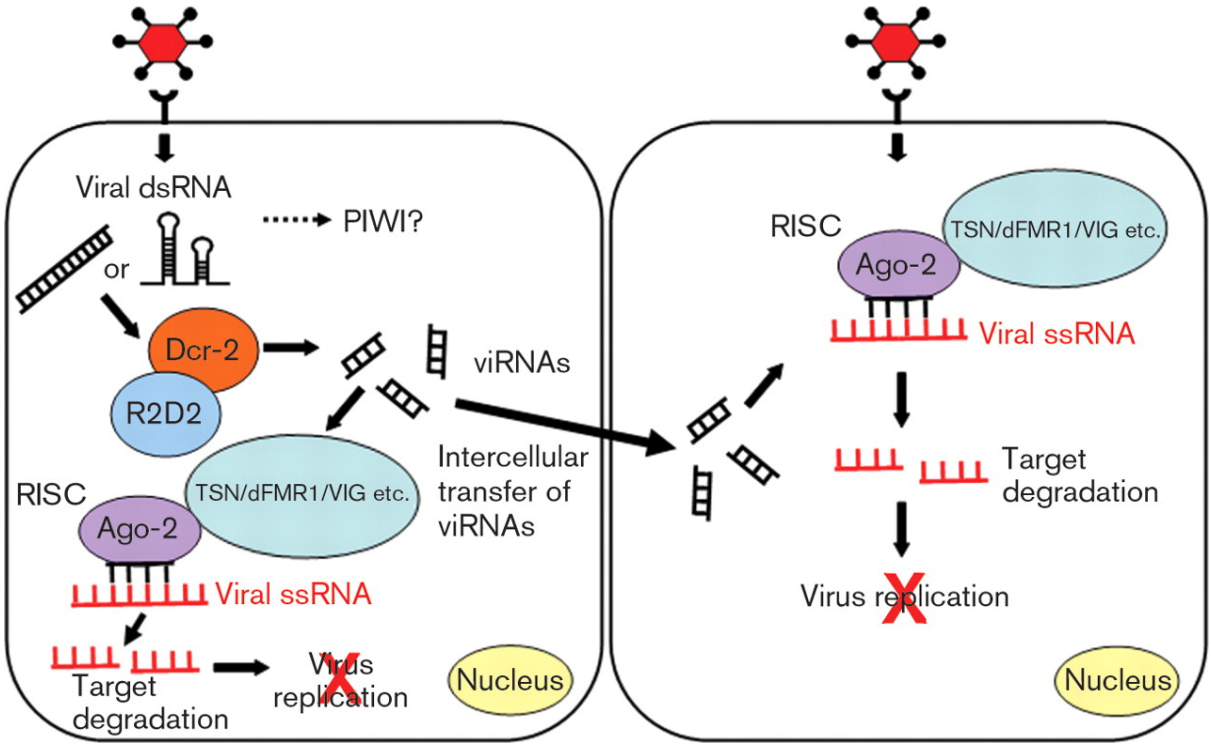

Fig.1.8. Antiviral siRNA pathway in mosquito cells. Once a virus enters the cell it triggers the RNAi pathway through dsRNA in the form of replication intermediates (in the case of Alphavirus and Flaviviruses). dsRNA is then processed by Dicer-2 into viRNAs which can be exported to neighbouring cells or processed by RISC complex. Once complementary ssRNA from viral genomes are found, the target transcript is degraded. From (Fragkoudis et al., 2009).

Arbovirus counteraction of immune pathways

Arboviruses have a relatively small genome with a limited number of proteins. The function of viral proteins, especially those of non-structural proteins in the case of flaviviruses and alphaviruses, are still unknown in mammalian and mosquitoes. Most of these proteins are multifunctional and it has been found that some of them interfere with the mosquito innate immunity pathways.

The ns5 protein of JEV is able to inhibit the STAT1 cascade and TYK-2 phosphorylation, therefore blocking interfering STAT signalling in vertebrate cells (Lin et al., 2006). In *Ae. albopictus* derived C6/36 cells the ns5 of JEV also blocks the tyrosine phosphorylation of STAT (Lin et al., 2004b). SFV infection of *Ae. albopictus* U4.4 cells inhibits signalling and DENV infection of *Ae. aegypti* mosquitoes inhibits Toll pathway signalling (Fragkoudis et al., 2008b; Sim and Dimopoulos, 2010).

As mentioned before, RNAi is the most important pathway in controlling arbovirus infection in mosquitoes. Some researchers have speculated that of a protein RNAi inhibitor existed in arboviruses, as described in true insect viruses, however, to the day, no inhibitor of the sort has been found. Infection of a mosquito or mosquito cell lines with an alphavirus expressing a true insect virus RNAi inhibitor, such as B2, which binds to dsRNA, induces cytopathic effects and increased mortality of the vector (Cirimotich et al., 2009). RNAi may to be the mechanism which holds the balance between persistent and cytopathic infection. It is not in the interest of the arbovirus to severely harm or kill the vector, therefore arboviruses are not known to express any protein RNAi inhibitors. On the other hand, like most relations in nature, the arbovirus-vector interaction is not stationary, and arboviruses may employ different strategies to slightly overcome RNAi and increase virus replication, titre and possibly vector infectivity. No arboviral RNAi inhibitor protein has been found, however, recently, it has been discovered that several flaviviruses have highly structured 3’ UTRs which act as RNAi inhibitors when cleaved from the viral genome (Dr. Esther Schnettler, personal communication). In SFV infection of U4.4 cells, the viRNAs produced map along the whole virus genome, however some places in the genome produce more than other. These are designated hot spots. Other parts of the genome produce a much lower number viRNAs, these are called cold spots. viRNAs produced from hot spots have a lower effect silencing SFV through the cellular RNAi machinery than those less abundant viRNAs from the cold spots. It is suggested that less efficient hot spot viRNAs act as a decoy saturating the system

(Siu et al., 2011b). This inhibition would lightly shift the balance to a higher virus production without the drastic negative effect of a true insect virus RNAi inhibitor peptide.

Apoptosis in mosquito cells

Apoptosis is programmed cell death, this mechanism is important in both development and as an innate antiviral response in vertebrate cells to limit virus replication and spread (Best, 2008). It is unknown if apoptosis plays an important role against arbovirus infection in mosquitoes and mosquito cells. In several cases, virus-induced pathology and apoptosis have been reported in infected mosquitoes (Girard et al., 2005; Girard et al., 2007; Mims, Day, and Marshall, 1966; Weaver, Lorenz, and Scott, 1992; Weaver et al., 1988). In DENV infected mosquitoes it has been shown that there is a differential regulation of enzymes involved in apoptosis (Xi, Ramirez, and Dimopoulos, 2008). However, to date, there is no conclusive data on the role of apoptosis in mosquito antiviral defence. Mosquito cell lines infected by arboviruses usually lack cytopathic effects and infection results in persistence and survival of the culture. In *Ae. albopictus* derived U4.4 cells, the growth rate of the culture does not change even if infected with SFV (Fragkoudis et al., 2008c). Cytopathic effects reported in mosquito cell lines seem to depend on particular virus strains and cell line clones (Condreay and Brown, 1988; Sarver and Stollar, 1977; Stalder, Reigel, and Koblet, 1983). It remains unknown whether mosquitoes lack the pathways to activate apoptosis upon infection or if arboviruses inhibit this pathway. Some of the core apoptotic genes in *Ae. aegypti* have been identified and it has been noted that the caspase family has been expanded as compared to drosophila (Liu and Clem, 2011). Several studies have been done with SINV expressing drosophila and *Ae. aegypti* pro-apoptotic genes, such as Michelob-x (Mx) or Reaper (Rpr) in mosquitoes (Wang et al., 2008a). Silencing of the *Ae. aegypti* anti-apoptotic gene iap1 (Aeiap1) with dsRNA caused apoptosis in midgut epithelium and enabled SINV to spread faster. However, silencing *Ae. aegypti* droc, an apoptosis inducer, had the opposite effect (Wang et al., 2012). These results suggest that SINV cannot inhibit apoptosis if this pathway is triggered by a pro-apoptotic gene. In cell culture, the initial viral production was not reduced when expressing pro-apoptotic genes, although, later reduction in viral titre was due to cell death. In the mosquito, artificially induced apoptosis in the midgut would disrupt three of the physical barriers that control arbovirus infection, the midgut, the matrix and the basal lamina, thus allowing a faster virus spread through the mosquito. Since the integrity of those cell walls are important and the virus can induce cell motility and tissue rearrangement the role of apoptosis remains circumstantial. Interestingly, the expression of an RNAi inhibitor

such as B2 by an alphavirus causes cytopathic effects in mosquitoes and mosquito cell culture (Cirimotich et al., 2009).

In arboviruses apoptosis does not appear to play a role in the maintenance of a persistent infection (Karpf, Blake, and Brown, 1997). Other control mechanisms, such as RNAi might be a useful trade-off for both parties; the virus and the mosquito, before inducing cell death or other *in extremis* mechanisms.

Limitations in mosquito research and future perspectives

Very little is still known about mosquito innate immune responses against arboviruses. Compared to other arthropod research, such as in drosophila, the number of tools available is very limited for mosquito research; only a few mutant aedine mosquitoes are available and very few antibodies or molecular reagents. Most work done in cell culture is based on *Ae. albopictus* cell lines derived from Singh, while *Ae. aegypti* mosquitoes are used for *in vivo* studies (Fragkoudis et al., 2009). Even though the genomes of *A. gambiae*, *Ae. aegypti*. and *C. quinquefasciatus* have been sequenced, their annotation is still quite poor and it will take several years until most of the genes and their functions are properly annotated. In the case of *Ae. albopictus* genome is not available yet. This vector has become of great importance in Europe after recent epidemics and it will probably become important in the Americas during this decade, therefore its genome sequence would be a helpful tool to the arbovirus community. Unfortunately, there is also a lack of cell lines from other mosquito and insect species, such as midges, that would allow comparison of viral replication dynamics under different host conditions.

In the last couple of years, several more developments have been presented. The use of transgenic aedine mosquitoes as tools has been described. Genetically engineered mosquitoes have been created expressing parts of the DENV genome as dsRNA, making them refractory to this arbovirus (Franz et al., 2009), as well as mosquitoes with altered innate immunity which can be useful tools for research (Zou et al., 2011). A new field has emerged in arbovirology with the discovery of the bacterium Wolbachia as an antiviral factor in insects (Hedges et al., 2008). Surprisingly, this bacterium does not naturally infect *Ae. aegypti* and *A. gambiae* mosquitoes, however, if artificially infected with laboratory strains of this bacteria, DENV replication in the midgut becomes inhibited (Bian et al., 2010) although mosquito mortality significantly increases (Evans et al., 2009; McMeniman et al., 2009). This discovery has opened the door to successful cage field trials in which *Ae. aegypti* mosquito populations become less susceptible to DENV (Hoffmann et al., 2011; Walker et al., 2011).

Recent research has also started investigating the antiviral role of other arthropod innate immunity pathways other than the classical Toll, Imd, JAK/STAT and RNAi. For example, the role of the gene *vago* has awakened some interest. This protein is highly up-regulated upon DCV infection in drosophila (Dostert et al., 2005). Mutants for *vago* also show a higher level of viral replication (Deddouche et al., 2008). Induction of *vago* is triggered by the DExD/H-box helicase domain of Dcr-2, without the involvement of Toll, Imd, Jak/Stat or

other members of the RNAi pathway. It has been suggested that Dcr-2 acts as a sensor for dsRNA which then triggers Vago and mounts an antiviral response, similar to that of RIG-I and MDA-5 in mammals. However, the mechanisms of this response are unknown (Merkling and van Rij, 2012). Other mechanisms, such as autophagy, might play a role in insect antiviral innate immunity, although its roll is still unclear since this pathway might cross talk with Toll (Nakamoto et al., 2012). However, this relation so far has only been found in drosophila and not in mosquitoes. The heat-shock response has been associated with ONNV infection of *A. gambiae* mosquitoes (Sim et al., 2005). Hsp70B is a chaperone protein which has been associated with the control of viral infection in the mosquito. Chaperons Hsp23 and Hsp27 have been found to have the same role in drosophila S2 cells infected with FHV (Go et al., 2006). Heat-shock proteins might be considered a danger signal that initiates innate cellular immune responses. Intriguingly, the Hsp70/90 machinery is critical in loading of siRNAs into the RISC in drosophila (Iwasaki et al., 2010). It has been hypothesised that induction of Hsp70 upon virus infection facilitates loading of the RISC with viRNAs therefore strengthens the antiviral efficiency of RNAi.

There are several innate immunity responses that could have an antiviral role but have not been investigated. In this thesis alternative antiviral innate immunity pathways, such the phenoloxidase cascade, will be thoroughly investigated with the help of transcriptomic studies.

Hypothesis

SFV infection of mosquito cells is controlled by uncharacterised innate immune mechanisms such as the melanisation cascade.

Aims

- To determine and identify yet uncharacterised innate immune responses of *Ae. albopictus* derived U4.4 cell line to SFV infection.
- To characterise the role of the PO cascade in arboviral infection of mosquitoes.

Chapter 2. Materials and Methods

Bacterial protocols

Bacterial culture

Two strains of Escherichia coli (*E. coli*) were used; SURE competent cells (Stratagene) and DH5-α cells (Invitrogen). Bacteria were grown in Luria-Bertani (LB) medium or on LB plates with 1.5% agar. Agar and media were both sterilised by autoclaving and the appropriate antibiotics (ampicillin (100µg/ml) or kanamycin (50µg/ml) were added for selection purposes prior to use. Agar plates were prepared by pouring melted LB agar onto 10 cm Petri dishes (Nunc). Transformed bacteria were streaked on the cooled agar surface with a glass rod. Plates were allowed to dry, inverted and incubated overnight at 37° C. The whole process was done under aseptic conditions in a CL1 cabinet or with a Bunsen burner. Single colonies were picked from the plates and inoculated into LB medium with the appropriate antibiotic. Flasks were then incubated overnight at 37° C with constant shaking (225 rpm). Cells were not allowed to grow for more than 16 h. In some cases, some of the viral proteins from plasmids expressed in bacteria could be toxic to cells. The expression of these proteins in bacteria was avoided by growing them in Soy Broth media (Sigma-Aldrich).

List of plasmids

pAcIE1-R*luc*	Provided by Dr. Esther Schnettler (Ongus et al., 2006)	Ampicillin Resistant
pSpe6-SFV4(3H)*RLuc*	Provided by Prof. Andres Merits	Ampicillin Resistant
CMV-SFV4-(*FFLuc*/H)-2SG-Egf1.0_F	Self provided (see chapter 5)	Kanomycin Resistant
CMV-SFV4-(*FFLuc*/H)-2SG-Egf1.0_R	Self provided (see chapter 5)	Kanomycin Resistant
CMV-SFV4-(nsP3-ZsGreen)-2SG-Egf1.0_F	Self provided (see chapter 5)	Kanomycin Resistant
CMV-SFV4-(nsP3-ZsGreen)-2SG-Egf1.0_R	Self provided (see chapter 5)	Kanomycin Resistant

Transformation of bacteria

SURE-competent cells (Stratagene) and DH5-α (Invitrogen) were transformed according to manufacturer's protocol. Briefly, a vial of cells was thawed on ice and 100µl of the bacteria suspension was aliquoted into 1.5 ml pre-chilled sterile tubes (Eppendorf). Approximately 200ng of plasmid was then added to the bacteria culture and incubated in ice for 30 min. Cells were then heat-shocked for 30 sec at 42 ° C followed by incubation on ice for 2 min. Nine hundred µl of Super Optimal broth with Catabolite repression (SOC) medium (Sigma-Aldrich) pre-warmed at 30° C was added to the mixture. The tubes were then incubated for 1 h on an orbital shaker (225 rpm) at 30° C in order to allow the bacteria to express the antibiotic resistance genes from the plasmid. Agar plates were then prepared with the appropriate antibiotic (ampicillin -100µg/ml, or kanamycin -50µg/ml) as described previously. 10, 50 or 100 µl of the transformed bacteria cells were pipetted onto the agar plates and evenly spread using sterile glass rods. Positive control transformation was monitored using the provided pUC19 plasmid. Once dried, plates were inverted and incubated at 30° C overnight (16 h). Colonies on the plates were examined 24 h later. A single colony was used to prepare DNA minipreps and maxipreps as described later.

Preparation of glycerol stocks

Frozen stocks of transformed bacteria were made in order to avoid regular transformation of bacteria when making preps. A single colony was isolated from an agar plate and used to inoculate 5ml of LB medium containing the appropriate antibiotic. The culture was grown overnight (16 h) in an orbital shaker at 30^0 C. Next day the bacteria were pelleted by centrifugation (5 min at 1500 x g) and the pellet resuspended in 1 ml of 50% sterile glycerol in H_2O. The sample was then transferred to a sterile eppendorf tube and snap frozen using dry ice. The tube with the transformed bacteria was then stored at -80^0 C and when required inoculums were obtained from it without allowing it to thaw.

Cell culture

Mosquito cell lines

All the mosquito derived cell lines were maintained in sterile plasticware (Nunc) at 28^0 C in an incubator and when handled all the procedures were done in a class II biological safety cabinet under sterile conditions.

Maintenance of mosquito cell lines

U4.4 cell lines were kept in L-15 medium (Leibovitz) containing 10% foetal calf serum (FCS)(Invitrogen), 10% tryptose phosphate broth (TPB) and antibiotics (penicillin/streptomycin – 100U/ml and 100 µg/ml, respectively) in 175 cm^2 tissue culture flasks (Nunc). These flasks were kept at 28^0 C in absence of CO_2. To passage the cells, old medium was removed from the tissue culture flask and 10 ml of fresh L-15 medium was added. The cells were dislodged with a cell scraper (Falcon) and the suspension was transferred into a universal tube. The cells were counted by using 10 µl of the suspension, diluting it 1/10 with trypan blue and using a haemocytometer. The required number of cells was then seeded into fresh culture flasks. Cells were passaged indefinitely.

Baby hamster kidney (BHK-21) cells

Baby hamster kidney (BHK)-21 cells were used to propagate and titrate viruses. Cells were maintained in 175 cm^2 tissue culture flasks (Nuclon, UK) in Glasgow's minimum essential medium (GMEM)(Gibco), supplemented with 10% (volume/volume (v/v)) new born calf serum (NBCS) (Invitrogen, UK), 10% (v/v) tryptose phosphate broth (TPB) (Invitrogen, UK), antibiotics (penicillin/streptomycin -100U/ml and 100µg/ml, respectively) and L-glutamine (200mM, Merk). Flasks were kept at 37^0 C in a humidified environment containing 5% CO_2. BHK-21 cells grow at an optimum pH of 7.2 to 7.4; therefore, a pH indicator (phenol red) is incorporated into the growth medium allowing the visual monitoring of the pH during cell growth. The culture medium turns yellow when the pH is low and pink when it raises.

Maintenance of mammalian cell lines

When cells were at 80-90% confluency, the culture medium was removed and the cell monolayer was rinsed with sterile PBS (sPBS). After sPBS was removed, 5ml of 0.05% trypsin/EDTA (Invitrogen) were added and the cells were incubated at 37^0 C until detached from the plastic. The trypsin mixture was then diluted in an equal volume of media to inactivate the enzyme. The cells were then concentrated at the bottom of a universal tube by centrifugation at 400 x gravity (g) for 5 min. The pellet was then resuspended in 10 ml of GMEM medium. 10 µl of this was diluted 1/10 with trypan blue and a haemocytometer was used to count the cells. The required number of cells was then seeded into fresh culture flasks.

Freezing and thawing of cells

Frozen stocks of all cell lines were prepared as following, cells were frozen when they were healthy and in the middle of a growth phase, but never more than 80% confluency. The cells were scrapped (in the case of mosquito cells) or trypsinised (in the case of mammalian cells) then counted using a haemocytometer. The optimal concentration of mosquito cells for freezing was 7×10^6 cells per ml and, in the case of mammalian cells it was 5×10^6 cells per ml. Once the cells were counted they were pelleted by centrifugation. The medium was then discarded and the cells were resuspended in freezing medium (of 10% dimethyl sulfoxide (DMSO) and 90% FCS). 1 ml of this suspension was then placed into 1.5 ml cryovials (Nunc), the tubes were then placed in a freezing box (Mr. Frosty, Sigma) containing isopropanol, and the box was placed into a -80^0 C freezer. The isopropanol coat allowed the cooling of the cells at approximately 1^0 C per min. The vials were left at -80^0 C overnight. After 16 h, the cryovials were transferred to a liquid nitrogen storage container. Liquid nitrogen cools the cells below -130^0 C, thus preventing the formation of damaging ice crystals.

When resurrecting cells from liquid nitrogen, it was crucial to warm them up as quickly as possible. Ahead of removing the cells from the liquid nitrogen environment, growth medium was put into a 25 cm^2 flask (Nunc), while another 10 ml of warmed medium was put into a universal. The cells were then transported on dry ice from liquid nitrogen to avoid defrosting. Prior to warming the cryovials, the caps were loosened slightly to release any gases and pressure it might have built up. The cryovials were then placed into a 37 ° C water bath to

allow a rapid defrosting of the cells then, mixed gently and transferred to a universal with pre-warmed medium. The cell suspension was centrifuged at 450 x g and the supernatant was decanted. The cells were resuspended in 10 ml of fresh medium, then transferred to a 25 cm^2 tissue culture flask and incubated in a humidified environment at 37^0 C with 5% CO_2 in the case of mammalian cells, or in a 28^0 C incubator for the mosquito cells. All tissue culture procedures were carried out in Class II biological safety cabinet to avoid contamination.

Mosquitoes

Rearing of mosquitoes

4-5 days old adult *Aedes aegypti* mosquitoes (a Liverpool red eye strain optimised for filarial growth) were kindly provided by R. Maizels (Institute of Immunology and Infection Research, School of Biological Sciences, University of Edinburgh). Live mosquitoes were kept in a stockinette sleeve openable cage (mesh panels on all sides) and reared at 27˚ C, in 85% humidity and with a 16/8 h light:dark photoperiod (Fig.2.1). To maintain the mosquitoes, a sugar meal was given by placing cotton wool soaked in 10% w/v fructose sugar solution on the top of the mosquito cage. Mosquitoes were one or two days old when provided, they were not allow to reproduce and they were not kept for more than 3 weeks. All the mosquito rearing and experiments were done in a containment level II insectary.

Fig.2.1 Mosquito rearing container

In order to use the mosquitoes for experiments, the insects were caged in waxed paper cartons during and after infections. The feeding cartons were modified to allow a one way entry point and a semi-accessible area for feeding. The entry point was created by cutting a small square hole (approx. 2 cm^2) at the side of the carton, which was taped over with two layers of elastic latex (cut from latex gloves). A small incision was cut on each of the layers (approx. 5mm). The lid of the carton was replaced with nylon mesh and sealed with multiple bands (Fig.2.#). Females were isolated from the main mosquito cage by suction through a plastic pipette containing two consecutive tubes attached to one of the ends. The pipette also contained 2 layers of nylon mash (one between the pipette and the first tube, the second one between both tubes) to avoid the swallowing of mosquitoes while suctioning. Once the females were aspirated into the pipette, they were quickly transferred into feeding container. This was achieved by introducing the pipette mouth through the little hole in the latex and expelling air. No more than 25 female mosquitoes were kept in these containers.

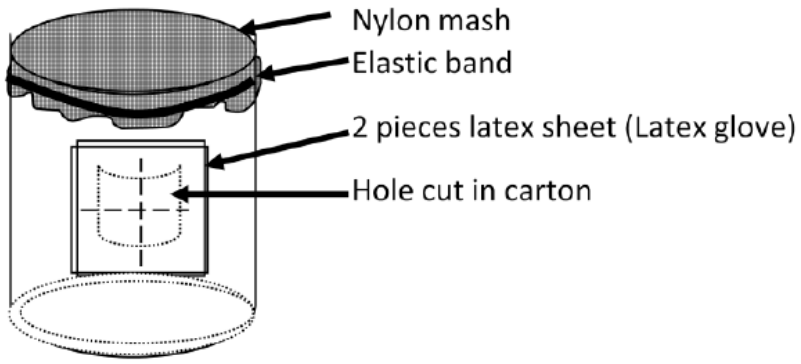

Fig.2.2. Mosquito carton. From Ricky Siu's PhD thesis.

Distinguishing between male and female *Ae. aegypti* mosquitoes

Only female mosquitoes were used in this project. There are several morphological characteristics that allow differentiation of female and male mosquitoes. Females are generally of a larger size and they have a larger abdomen. Males have a smaller size and the flagelomere (hairs protruding from their antennae) are longer, giving their antennae a feather-like look (Fig.2.3).

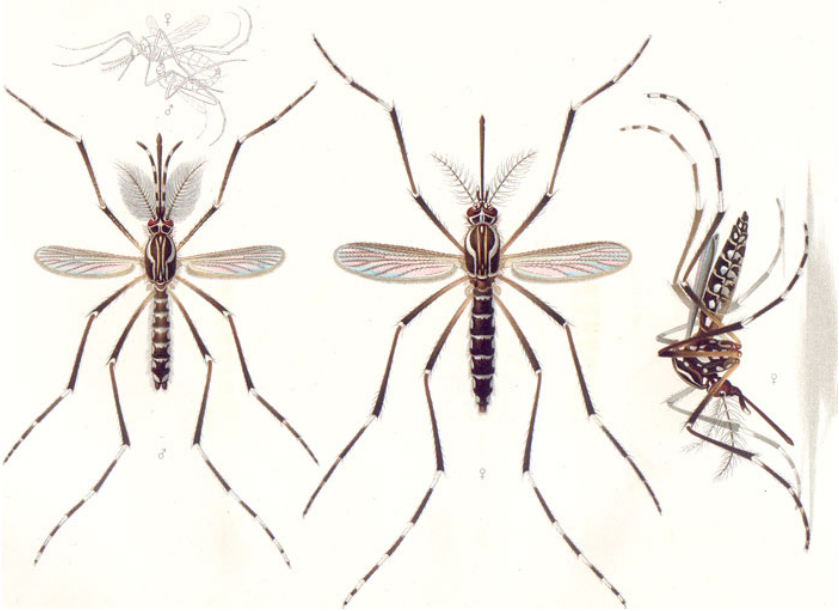

Fig.2.3. Differences between female and male *Ae. aegypti* mosquitoes. From left to right: male, female and feeding female. Adapted from (Goeldi, 1905).

Infection of mosquitoes through a blood-meal

The mosquito infectious blood-meal consisted of defibrinated ovine blood (TCS Biosciences) which was mixed with virus (5 x 10^7 PFU/ml) and supplemented with 4 mM ATP. The blood-meal was placed in a Haemotek membrane feeder (Discovery Workshops, Accrington, UK, hemotek@discoveryworkshops.co.uk) to enable the feeding. A collagen membrane provided with the machine was used. The Haemotek terminal was placed on the top of the nylon mash and the mosquitoes were allowed to feed for 2 h by biting through the mesh. After the feeding took place fed mosquitoes were selected for the experiment, the unfed mosquitoes were discarded and submerged in ethanol. Selection was achieved by placing the feeding containers into cold chambers, generally ice boxes, for 10 min. This will anaesthetise the insects and allows handling carefully with forceps. Once the selection was completed, the

feeding container was sealed once again and moved to a 28^0 C incubator inside the insectary. The blood-meal infection and handling of fed mosquitoes always took place in a glove box inside the insectary.

Nucleic acid techniques

Plasmid DNA extraction from transformed bacteria cultures

Minipreparation of DNA

DNA plasmids were purified on a small scale using a Miniprep kit (Qiagen) according to the manufacturer's instructions. In brief, inoculum from the transformed bacteria colony was cultured in a volume of up to 10 ml in a universal at 37^0 C as described before. The bacteria were then pelleted by centrifugation at 1500 x g for 5 min and the liquid discarded. The bacteria were then resuspended in 250 µl of buffer P1 and transferred into a 1.5 ml tube (Eppendorf). Cell lysis was achieved by adding the provided lysis buffer, P2. This alkaline buffer solubilises the bacterial membrane and disassociates DNA and proteins. The mixture was gently mixed thus avoiding damage to genomic DNA. The reaction was allowed to occur for a couple of minutes then 350 µl of buffer N3 was added to neutralise the reaction and enable precipitation of protein and membrane lipids. The mixture was then centrifuged at 17,900 x g for 10 min. After centrifugation the supernatant was transferred into a Qiaprep mini column and centrifuged at 17,900 x g for 1 min. The flow-through was then discarded and the filter was washed with 750 µl of PE buffer and centrifuged once again at 17,900 x g for 1 min. The flow-through was again discarded and the filter was centrifuged at 17,900 x g for a further 2 min to allow drying. The plasmid DNA bound to the column was eluted by adding of 30 µl of RNase and DNase free water directly onto the centre of the column and allowing it to stand for 1 min. Eluted DNA plasmids were collected by placing the filter column in a 1.5 ml tube (Eppendorf) and centrifuging at 17,900 x g for 1 min. The plasmid DNA was then quantified and stored at -20^0 C.

Maxipreparation of DNA

Maxipreps were used to purify large amounts of plasmid DNA from large volumes of transformed bacterial cultures. They were carried out using an endotoxin free maxiprep kit (Qiagen). Single colonies of transformed bacteria were inoculated into 250 ml of LB broth containing the appropriate antibiotics. After 16 h of growth at 37^0 C the bacteria were

harvested by centrifugation at 6000 x g for 20 min at 4^0 C. The liquid was discarded and the pellet was thoroughly resuspended in 10 ml of buffer P1 then transferred to 50 ml tubes (Falcon). As with the miniprep, the bacteria cell was were broken by adding 10 ml of P2 lysis buffer and gently mixing it by inverting the tube 4-6 times. The mixture was allowed to stand for 5 min at room temperature. In order to neutralise the reaction, 10ml of buffer P3 were added and mixed gently. This was then poured into a Qiafilter cartridge and the precipitate formed in the previous stage was allowed to settle at the top of the cartridge for 10 min. Then the lysate was pushed through the filter in a 50 ml tube (Falcon). Two and a half mls of ER buffer was then added to the flow through and it was mixed thoroughly by inverting 10 times then placed on ice for 30 min. During incubation, the Qiagen-tip 500 was equilibrated by allowing 10 ml of buffer QTB to flow through it. After the 30 min incubation, the sample was poured into the Qiagen tip-500 and allowed to flow through, to bind the plasmid DNA to the membrane. The filter was then washed twice with 15 ml of buffer QC and the plasmid DNA eluted by passing 15 ml of buffer QN though the filter. In order the precipitate the DNA, 10.5 ml of room temperature isopropanol was added and the mixture centrifuged at 15,000 x g for 30 min. The precipitated plasmid DNA formed a pellet at the bottom of the tube and was washed with 70% ethanol prior to centrifugation at 15,000 x g for 10 min in order to remove traces of isopropanol. The supernatant was poured off at the end of this centrifugation and the pellet was allowed to dry at room temperature. The plasmid pellet was then redissolved in 500 µl of endotoxin free TE buffer (Qiagen).

Restriction enzyme digestion

In order to perform restriction enzyme digest, up to 1 µg of plasmid DNA were digested with 1 unit (U) of enzyme. The enzyme unit (U) indicates the amount of a particular enzyme that catalyses the cleavage of 1 µg of DNA at its optimum temperature in 1 h. The restriction enzyme digests were carried out in a final volume of 20 µl, and contained 2 µl of 10X restriction enzyme buffer, 2 µl of 10X acetylated bovine serum albumin (BSA), 1 µl of enzyme (1 unit/µl), plasmid DNA, and DNase free H_2O made up to a final volume of 20 µl. The reactions were incubated at the optimum temperature for 2-4 h. The restriction products were analysed using agarose gel electrophoresis and purified for further manipulation (See below).

Purification of DNA after restriction digestion

DNA fragments were purified after restriction digestion using a Jetquick DNA purification kit (Genomed). The volume of the restriction digest was brought up to 100 µl with DNase-free H_2O. Then 400 µl of binding buffer H1 was added. The mixture was transferred into the binding column and centrifuged for 1 min at 12,000 x g. The flow-through was then discarded and the column was washed twice with washing buffer H2. Then the column was allowed to dry by centrifugation once more in order to remove residual washing buffer. The DNA fragments were eluted by adding 30 µl of DNase-free H_2O preheated to 60^0 C directly onto the binding matrix. The column was then allowed to stand for 1 min at room temperature. The eluted DNA was collected by centrifugation at 12,000 x g into a 1.5 ml DNase/RNase-free tube and stored at -20^0 C.

***In vitro* transcription of capped infectious DNA**

1.66 µg of *Spe*I linearised infectious DNA plasmid was used in the transcription reaction. The RNA was synthesised at 37^0 C for 2 h, using the cap analogue m7G(5')ppp(5')G (GE Healthcare) to produce capped viral genomic RNA transcripts. The composition of the reaction is the following:

*Spe*I cut plasmid (1.66µg)	xx µl
10X SP6 buffer (GE Healthcare)	5.0 µl
10mM M 7 G (5')ppp (5') G (cap)(GE Healthcare)	5.0 µl
50 mM DL Dithiothretiol (Sigma)	5.0 µl
rNTP mix (10 mM ATP, CTP and UTP, 5 mM GTP)(GE Healthcare)	5.0 µl
H20 (Ambion, nuclease free)	x.x µl
Recombinant RNasin Ribonuclease Inhibitor (60 U/1.5µl)(Promega)	1.5 µl
SP6 RNA Polymerase (50 U/µl)(GE Healthcare)	1.5 µl
Total Volume	50.0 µl

Verification of synthesised viral genomic RNA was achieved by agarose gel electrophoresis. RNA transcripts were generally used immediately, but when stored they were kept at -80^0 C.

Agarose gel electrophoresis

RNA and DNA fragments were separated in gels containing 1-1.5% agarose (depending on fragment size) in 0.5M tris-borate (TBE) buffer with 0.5 µg/ml ethidium bromide to enable

visualisation of the nucleic acids under UV light. Samples were mixed with 6X loading buffer (NEB) and loaded in the wells of the agarose gel. Then an electric current was applied to the gel (70-130 volts depending on the percentage and size of the gel) for approximately 1 hour in order to separate different fragments of nucleic acids. As a size marker, two different DNA ladders were used; 1 Kb and 100 bp ladders (Promega). Nucleic acid fragments were visualised using a UV transluminator.

DNA extraction from agarose gel

DNA fragments were extracted from agarose gels using the PCR purification kit (Roche) according to manufacturer's instructions. Briefly, DNA fragments were separated by electrophoresis and a long wavelength UV transluminator was used to visualise the fragments. Using a sterile scalpel the fragments were excised from the agarose gel, placed in a tube and weighed. 750 µl of binding buffer were added and the sample was incubated at 50^0 C with occasional vortexing until completely dissolved. The sample was then applied to a prewarmed filter column and centrifuged for 1 min at 17900 x g to bind the DNA onto the silica membrane. The flow-through was discarded and the column washed twice with 500 µl of washing buffer. The column was then dry centrifuged at 17900 x g to enable the removal of all of the washing buffer. 30 µl of DNase-free water was used to elute the DNA by applying them on the silica membrane and allowing it to stand for 1 min at room temperature. The DNA was then collected by centrifugation of the column and stored at -20^0 C until required.

Nucleic acid quantification

All the nucleic acid samples (DNA, ssRNA and dsRNA) were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). One µl of nucleic acid sample was placed on the lens of the NanoDrop and the quantity and quality were measured.

Polymerase chain reaction (PCR)

Specific primers and reaction cycles were used are listed below. Two different PCR reactions were made depending on the length of the product. The PCR reaction mix had a final volume

of 20 µl, and contained 2 µl of 10X PCR buffer (15mM $MgCl_2$ included), 1 µl dNTPs (10 mM)(Promega), 0.5 µl of each primer, forward and reverse (50pM)(Sigma-Aldrich), 0.3 µl of *Taq* polymerase (Promega), DNA template and RNase/DNase-free water to a final volume of 20 µl. The reactions were incubated in a thermal cycler (Veriti®, Applied Biosystems) using the following settings: an initial denaturation stage of 2 min at 95^0 C, followed by an amplification stage which consisted of 25-30 cycles in 3 steps; denaturation of the template at 94^0 C for 30 seconds, annealing at X^0 C for 30 seconds and extension at 72^0 C (duration depending on the length of amplified region); then a final denaturation of the enzyme at 72^0 C for 15 min. A negative (no template) and a positive (β-actin) control were used to test for possible contamination and to confirm reaction functionality. The PCR products were examined by agarose gel and stored at -20^0 C.

List of primers

Name of primer	Primer sequence (5'-3')	Melting temperature (mT) used
Egf1.0_BamHI F	agGGATCCATGTCGAACAACATTTTCCTG	65^0 C
Egf1.0_BamHI R	agGGATCC**CTA**ATCAAGAGTTTCTTGATCGATTATTC	65^0 C
Serpin A External F	5´ CCTAACGgCAGAGGAAATGT 3´	55^OC
Serpin A External R	5´GCGCCTTCTTCATTGACTTC3´	55^OC
Serpin A T7 F	5´TAATACGACTCACTATAGGGAGACGGCAATAAGAGCCTCA3´	55^OC
Serpin A T7 R	5´GTGCACAACCTTGGAAACCTGGGATATCACTCAGCATAAT3´	55^OC
Serpin B External F	5´ATTTCGCCATTTTCCATCAG3´	55^OC
Serpin B External R	5´GTGCACAACCTTGGAAACCT3´	55^OC
Serpin B T7 F	5´TAATACGACTCACTATAGGGAGACGGCAATAAGAGCCTCA3´	65^OC
Serpin B T7 R	5´GTGCACAACCTTGGAAACCTGGGATATCACTCAGCATAAT3´	65^OC

Serpin C T7 F	5´TAATACGACTCACTATAGGGCggtGTACTACAAGgCGAAg3´	55^{O}C
Serpin C T7 R	5´CATAGTAGACTTGtCCTTTTGATATGGCGGGATATCACTCAG CATAAT3´	55^{O}C
Serpin D Ext F	5´CTGGAGATTGCCACCAAGTT3´	55^{O}C
Serpin D Ext R	5´AAGAACAAGAAGGGCCGATT3´	55^{O}C
Serpin D T7 F	5´TAATACGACTCACTATAGGGCCACGGTTGACAAAGTTCCT3´	55^{O}C
Serpin D T7 R	5´GCTGCATAGGCTTCACTTCGGGATATCACTCAGCATAAT3´	55^{O}C
Serpin E Ext F	5´CCAAGTATCAGATCATTTCaGATCA3´	59^{O}C
Serpin E Ext R	5´aTGGGACAATCGTCCATCTT3´	59^{O}C
Serpin E T7 F	5´TAATACGACTCACTATAGGGCCACGGTTGACAAAGTTCCT3´	60^{O}C
Serpin E T7 R	5´CGCtTCATTTGGTTGGATCTGGGATATCACTCAGCATAAT3´	60^{O}C
Serpin F Ext F	5´ AACAAtGcCCTGGAGATGAT3´	55^{O}C
Serpin F Ext R	5´tTGATaAACGTcCGCCaAAC3´	55^{O}C
Serpin F T7 F	5´TAATACGACTCACTATAGGGactcGCaGGTCGAACTCAAT3´	60^{O}C
Serpin F T7 R	5´CTCGACGACCtTCTCCaAAGGGGATATCACTCAGCATAAT3´	60^{O}C
Serpin G Ext F	5´ATTTCGTCGAAGCCGATCT3´	59^{O}C
Serpin G Ext R	5´ TGTGCTGCTGTTGGCtTACt3´	59^{O}C
Serpin G T7 F	5´TAATACGACTCACTATAGGGAGAAAGCCGAAAAaTCAGCA3´	65^{O}C
Serpin G T7 R	5´CGAAACGGAGAAAATGCTTCGGGATATCACTCAGCATAAT3´	65^{O}C
Serpin H Ext F	5´CAGCACACGCTTGCTATGTT3´	55^{O}C
Serpin H Ext R	5´CGTCCCAACTCGCATAGATT3´	55^{O}C

Serpin H T7 F	5´TAATACGACTCACTATAGGGAGCTCTCCATCGCCAaCTAa3´	60^{O}C
Serpin H T7 R	5´CCAAACACATTGAACGCATGGGATATCACTCAGCATAAT3´	60^{O}C
Serpin I Ext F	5´TTCCAGTGTGCAAAGCAAAG3´	55^{O}C
Serpin I Ext R	5´GGCGTTTTGtGTTCGATTTT3´	55^{O}C
Serpin I T7 F	5´TAATACGACTCACTATAGGGCTCGTTTTCAGCAGCATCAG3´	60^{O}C
Serpin I T7 R	5´AAAGTCCAGGTATTGGGTTTCAGGGATATCACTCAGCATAA T3´	60^{O}C
Serpin J Ext F	5´TGCCATTTACTTCAAGGGACT3´	57^{O}C
Serpin J Ext R	5´CCAGTCGATTCCTCCTCGAT3´	57^{O}C
Serpin J T7 F	5´TAATACGACTCACTATAGGGACCGTTCACTACCGCATTtC	67^{O}C
Serpin J T7 R	5´AGGAACGGACGATTCAcaAGGGATATCACTCAGCATAAT	67^{O}C
Serpin K Ext F	5´GGAATCTTCCAACGATCCTG	63^{O}C
Serpin K Ext R	5´ACTTTGAGGAACGCATCCTG	63^{O}C
Serpin K T7 F	5´TAATACGACTCACTATAGGGGGATCTTTTCCAAGCTGCTG	68^{O}C
Serpin K T7 R	5´CCGAGCTTCTTCAGTGGTTCGGGATATCACTCAGCATAAT	68^{O}C

Table.2.2. List of primers used in this study

Reverse transcription PCR

Reverse transcription PCR (RT-PCR) was used to create cDNA from mRNA templates using Oligo dT (NEB) as primer using SuperScript III kit (Invitrogen). Each reaction contained 1 µl 10 mM dNTP solution (Promega), 1 µl Oligo dT primer (500 µg/ml) (Promega), 4 µl of $MgCl_2$ (25mM), 5 µg of mRNA and dH_2O made up to a total volume of 10.5 µl. This mixture was incubated at 65^0 C for 5 min then cooled on ice for 2 min. The 4 µl of 5X first strand buffer (Invitrogen), 2 µl of dithiothreitol (DTT, 0.1 M) (Invitrogen), 0.5 µl RNaseIn

(Promega) and 1 µl of SuperScript III (Invitrogen) were mixed and combined with the previous mixture. This reaction was then incubated at 50^0 C for 1 hr. The reaction products were visualised on a 1% agarose gel.

Long dsRNA production

Long dsRNA transcripts were produced using MegaScript RNAi kit (Ambion) according to the manufacturer's instructions. This kit uses a T7 DNA dependent RNA polymerase, therefore all the primers used had a T7 sequence.

Total RNA extraction from cell monolayers

Total RNA was isolated from U4.4 cells using Trizol reagent (Invitrogen) according to the manufacturer's guidelines. Cells were grown on monolayers in 6-well plates. They were infected or mock-infected as indicated and after the required time the culture medium was removed, and 1 ml of Trizol was added into each well (approximately 1 ml per 10 cm^2). The cells were then scraped and the lysate was pipetted into a 1.5 ml RNAse free tube (Eppendorf). The lysate was passed through a 20 g syringe several times and left to stand for 5 min to allow the disassociation of protein and nucleic acid complexes. Following this, 200 µl of chloroform were added to the lysate and the mixture was shaken vigorously for 15 seconds. It was then allowed to stand at room temperature for 2 to 3 min before being centrifuged for 15 min at 12,000 x g at 4^0 C in order to precipitate membranes, fat, polysaccharides and high molecular weight DNA. The top aqueous phase containing the RNA was removed and transferred to an RNAse free 1.5 ml tube. 500 µl of molecular grade isopropanol (Invitrogen) was added to allow precipitation of RNA. The mixture was incubated at room temperature for 10 min and centrifuged at 12,000 x g for 10 min at 4^0 C. The supernatant was removed and the RNA pellet was left untouched at the bottom of the tube. This pellet was washed by addition of 1 ml of 75% molecular grade ethanol (7.5ml ethanol (Invitrogen) and 2.5 ml RNase-free water (Invitrogen)) and mixed by gently turning the tube, followed by 5 min of centrifugation at approximately 7.500 x g at 4^0 C. The supernatant was then discarded and the pellet was air dried for 10 min prior to resuspension in 50 µl of RNAse-free water and stored at -80^0 C.

Total RNA extraction from whole mosquitoes

Individual mosquitoes were placed in single 1.5 ml tubes in a cold chamber inside a glove-box. Those tubes were then labelled and dropped into a liquid nitrogen container where they snap froze. Plastic pestles were used to shatter the frozen mosquitoes and allow the extraction of RNA from the chitin rich insect body. The tips of the pestles were frozen before pressing the mosquitoes against the walls of the tube to enable keeping the body of the insect at a low temperature (brittle) thus making it easier to shatter. Trizol reagent (Invitrogen) was then used to extract the total RNA from the shattered mosquitoes as indicated above.

Transfection of cultured cells by electroporation

DNA in plasmid form or RNA from *in vitro* transcription was transferred into BHK-21 cells using a BioRad Gene pulser Xcell electroporator. On the day of the experiment, the cells were collected, counted and resuspended in ice-cold phosphate buffered saline (PBS) at a concentration of 6.25 x 10^6 cells per ml. Nucleic acids were mixed with 800 µl of cell suspension. Four hundred µl of the cells/nucleic acid mixture were added to a 0.4 cm electroporation cuvette, and cells were pulsed twice using a square wave of 140 volts for 25 milliseconds. Electroporated cells were transferred to 175 cm^2 tissue culture flasks containing fresh complete GMEM medium was added. The flasks were incubated at 37^0 C with 5% CO_2.

Transfection of cultured mosquito cells using liposomes

In order to prepare mosquito cells for transfection, 24-well plates were seeded with U4.4 cells in 1 ml of medium per well 24 h prior to transfection. At the time of transfection each well contained 1.3 x 10^5 cells. Nucleic acids such as plasmid DNA, dsRNA or ssRNA were adjusted to the desired concentration and diluted in Opti-MEM medium (Invitrogen) and mixed gently in a polystyrene tube. Following that, 1 µl of Lipofectamine 2000 (Invitrogen) was diluted into 50 µl of Opti-MEM medium in a separate polystyrene tube. The two mixtures were incubated separately for 5 min and then added together and incubated for further 20 min at room temperature. 100 µl of the Opti-MEM, nucleic acid and liposome mixture was added into each well. The plate was then incubated for 5 h at 28^0 C before the medium was replaced with fresh complete L-15 medium.

Virus techniques

All the recombinant viruses were made from cDNA plasmids kindly provided by Prof Andres Merits, (University of Tartu, Institute of Technology, Estonia). These plasmids were amplified by transforming chemically competent *E. coli*, growing cultures and harvesting the plasmids as described above. Two kinds of SFV plasmids were provided, one in which a CMV promoter transcribes an infectious SFV mRNA transcript directly from the plasmid following electroporation into the cells, and a second system in which an *in vitro* transcribed RNA with a cap analogue is required to produce infectious genomic SFV RNA. For the second type of SFV infectious DNA clone, the cDNA was linearised using *Spe* I and used as a template for *in vitro* transcription to produce capped viral RNA. Viral RNAs were then electroporated into BHK-21 cells. Virus was harvested in the supernatant at 24 and 48 h after electroporation. All the purified virus stocks were stored at -80^0 C.

Virus purification

48 h after electroporation of SFV *in vitro* transcribed RNA or SFV CMV plasmids into BHK-21 cells, the cultures were monitored for cytopathic effects. The supernatant was collected and clarified three times to remove cell debris by centrifugation (27,000 x g) at 4^0 C for 30 min, using a JA 20 rotor in a J2-21 centrifuge (Beckman Instruments). In order to pellet the virus, the clarified supernatant was ultracentrifuged through a 20% sucrose cushion. Aliquots of 17.5 ml of virus were added to Beckman SW28 ultracentrifuge tubes followed by addition of 20% (w/v) sucrose (Sigma) in TNE buffer pH 7.4 (50 mM Tris HCl pH 7.4, 100 mM NaCl and 0.1 mM EDTA pH 8.0, filter-sterilised) underneath the supernatant using a pipette until the tube was filled. Virus particles were pulled and purified through the sucrose cushion at 84,600 x g for 90 min at 4^0 C. The supernatants were then decanted from the ultra-centrifuge tube and the pellet re-suspended in 100 µl of TNE buffer on a rocker overnight at 4^0 C. The resuspended virus was then aliquoted into 1.5 ml tubes (Eppendorf). The centrifuge tube was then rinsed with 50 µl of TNE buffer in order to collect any remaining virus. The total pooled purified virus was aliquoted into 1.5 ml (Eppendorf) tubes (50 µl working stocks per tube) snap frozen on dry ice and stored at -80^0 C.

Virus infection of cells

When infecting cell monolayers virus was suspended and diluted in phosphate buffered saline (PBS) and BSA (PBSA, 0.75 g BSA per 100 ml PBS [0.75%]). The albumin proteins prevent virus binding to the charged plastic walls of the tube, pipette tip or other plastic surfaces. In

order to infect cells on a plate, the culture medium was removed from the wells and the required amount of virus was diluted into 450 µl (6-well plate) or 110 µl (24-well plate) of PBSA. 400 µl or 100 µl were added into each well, being careful not to disrupt the monolayer. The cells were then placed on a rocker at room temperature for an 1 hr to allow an even spread of the virus. After 1 h, the PBSA containing the virus was removed and the appropriate amount of fresh complete culture media was added into each well.

Titration of virus

SFV titres were determined by a standard plaque assay in BHK-21 cells (Strauss, Lenches, and Stamreich-Martin, 1980). Agar was prepared by mixing 4 g of Bacto-agar per 100 ml of PBS. This was then autoclaved to ensure it was free from contamination. BHK-21 cells were seeded with 2 ml of 10% GMEM at a density of 5 x 10^5 cells per well at the time of infection. Ten-fold serial dilutions of the samples or stock to be titrated were prepared using PBSA. Media from the wells was then removed and 400 µl of each dilution was placed into 2 wells of the 6-well plate, each dilution was done in duplicate. The plates were placed to rock for 1 h in a humid chamber. During the hour of rocking, the agar was melted in a water bath at 90^0 C then cooled to 55^0 C. GMEM medium with 2% NBCS (v/v) was prepared and warmed to 37^0 C. After the infection, the agar at 55^0 C was mixed with the 2% GMEM medium in a 1:3 ratio (agar: medium) by gently pipetting and the mixture was carefully added to the cell monolayers. After the agar hardened the plates were then incubated at 37^0 C and 5% CO_2 for 3 days.

After 3 days the cells were fixed with 10% neutral buffered formaldehyde (NBF)(Surgipath) by pouring it directly on top of the agar and leaving the plates at room temperature for at least 40 min. The NBF was then poured off and the agar plug was then removed using a metal spatula. Approximately, 1 ml of 0.1% toluidine blue was added into each well to stain the cells. The plates were then placed on a rocker for 1 hour to allow staining of the cell monolayer. The dye agent was poured off and the wells were gently washed with tap water. To calculate the plaque forming units (PFU) per ml content of each sample, the following formula was used:

$$\left(\frac{Average\ number\ of\ plaques}{amount\ of\ inoculum\ (ml)}\right) \times dilution\ factor$$

Fixing and visualisation of mosquito cells

In order to visualise fluorescent marker proteins such as ZsGreen cells were grown on glass coverslips and fixed with 10% neutral buffered formaldehyde for 45 min. The glass slide was then washed 3 times with PBS for 10 min each time. The converslip was drained before being mounted with fluorescent mounting medium, one converslip per microscope slide, and sealed with nail polish. The fixed slide was kept at 4^0 C in the dark. To check for the presence of ZsGreen fluorescence, a Nikon inverted microscope fitted with UV light (with excitation filter 485 and emission filter 530) was used in order to excite the green fluorescent protein. In order to determine infection rate or cytopathic effects in cell culture. Slides were checked under a Nikon inverted microscope. To check for the presence of ZsGreen fluorescence a Nikon inverted microscope fitted with UV light (with excitation filter 485 and emission filter 530) was used.

Detection of proteins on membranes

Isolation of protein for Western Blots

The cell lysis buffer consisted of 50 µl of β-mercaptoethanol (Sigma) mixed with 950 µl of Laemmli buffer (Bio-rad). Cells in a well of a 6-well plate were lysed by removing the culture medium and adding 200 µl of lysis buffer. The mix was then collected after 1 minute of incubation at room temperature and boiled for 10 min at 100^0 C. The samples were then frozen at -20^0 C until required.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

A 12% acrylamide resolving gel was made with 30 ml of 40% acrylamide (Sigma), 43 ml of dH_2O, 25 ml of 1.5M tris-base pH8.8 (Sigma), 1 ml 10% sodium dodecyl sulphate (SDS)(Sigma). The solution was polymerised following the addition of 5 µl of tetramethylethylenediamine (TEMED, Sigma) and 50 µl of 10% ammonium persulfate (APS, Sigma) were added to 5 ml of the solution and it was poured between the glass plates used to make the gel. A space of approximately 2 cm was left at the top for the stacking gel to fit. This space was filled with isopropanol (Sigma) while the gel set, to prevent air bubbles

forming and allow the gel surface to set flat and straight. To make the 4% stacking gel, 10 ml of 40% acrylamide (Sigma) was mixed with 64 ml of dH_2O, 25 ml 0.5M tris-base pH 6.8 (Sigma) and 1 ml of 10% SDS (Sigma). Fifty µl of APS (Sigma) and 5 µl of TEMED (Sigma) were again added to 5 ml of the previous solution to enable the polymerisation of the gel. The isopropanol was removed once the resolving gel had set and the stacking gel was poured on top. A comb was then inserted to make the wells. Once the gel had set it was placed in an electrophoresis tank which was subsequently filled with running buffer (see recipe below). An equal amount of protein solution was loaded into each well as well as a rainbow molecular weight marker (GE Healthcare). The gel was then run at 30 V for 30 min to allow the protein to enter and pass through the stacking gel slowly and then run at 130 V until the blue dye band reached the bottom of the gel.

Transfer of proteins from the SDS-PAGE gel to a nitrocellulose membrane

The gel was carefully removed from the scaffold and glass plates and the stacking gel was removed. The nitrocellulose membrane (Hybond ECL, GE Healthcare) was cut to the same size of the cell and soaked in H_2O for 10 min prior to the transfer. Whattmann papers (GE Healthcare) were soaked in semi-dry blotting buffer buffer. The transfer sandwich was then assembled starting with a piece of filter paper. The gel was then placed on top of the filter paper with the nitrocellulose membrane on top of the gel. The 'sandwich' was finished with another piece of filter paper on the top. This was placed in a semi-dry transfer apparatus. The proteins in the gel are negatively charged after mixed with the Laemmli buffer, so an electrical charge allows them to transfer to the positively charged nitrocellulose membrane. The transfer was run at 15 V for 30 min and the current at a maximum of 3 mA/cm^2 for 30 min. Once the transfer was complete, the sandwich was taken apart and the nitrocellulose membrane was placed in a petri dish with 2% blocking buffer (2% (w/v) milk powder in PBS+0.1% Tween) overnight at 4^{o} C or 1 hour at RT on a rocking plate.

Detection on membranes

After the nitrocellulose membrane finished blocking, it was washed with PBS + 0.1 % Tween (v/v) – 20 (PBS-T)(3 x 5 min). 10 ml 0.2% blocking buffer and primary antibody (eGF1.0 at 1:35000) Then the membrane was incubated with the primary antibody (Egf1.0 at 1:35000) diluted in 10 ml 0.2% blocking buffer for 1 h at room temperature. It was then washed with PBS + 0.1 % Tween – 20 (PBS-T)(3 x 5 min). Primary antibodies were detected by incubating the secondary antibody diluted 1:5000 in PBS-T for 1 h with the membrane (Goat

anti-rabbit-AP; Abcam, ab6722). It was then washed with PBS + 0.1 % Tween – 20 (PBS-T)(3 x 5 min). The protein band was visualised by adding 10 ml of AP-buffer and 200 µl of NBT/BCIP solution (Roche) and then incubating on a rocking plate until the band was visible. The reaction was stopped by washing the membrane in H_2O (3 x 5 min).

Western Blot Buffers

Semi-dry blotting buffer	Running buffer
39 mM glycine	25 mM Tris
48 mM Tris Base	250 mM glycine
0.037 % w/v SDS	0.1 % w/v SDS
20 % v/v Methanol	

All the materials required for the preparation of the transfer and running buffers were obtained from Sigma

Blocking buffer	AP Buffer
5% w/v blocking powder (GE Healthcare)	100 mM NaCl
PBS + 0.1% w/v Tween-20 (Sigma)	5mM $MgCl_2$
	100 mM Tris-HCl pH 9.5

Dual luciferase assay

A Dual-Glo Luciferase Assay System (Promega) was used to measure both *Renilla* (*RLuc)* and *Firefly* luciferases (*FFLuc*). Infected cell monolayers or infected whole mosquitoes were lysed by removing the media from the wells. The tissues were agitated for 5 min to enable the disruption of the cell membranes. 1 µl of the lysate was mixed with 70 µl of luciferase assay reagent and measured in a luminometer (Turner Designs, model TD-20/20) with a 10 sec

equilibration time and a 10 sec integration time. 70 µl of Stop & Glo reagent was then added to inhibit the *FFLuc* activity and determine *RLuc* activity.

Roche 454 Sequencing

A six-well plate with 6.5 x 10^5 U4.4 cells per well was infected with a multiplicity of infection of 10 PFU of SFV. A similar plate was mock-infected. Total RNA from infected and uninfected U4.4 cells was isolated using Trizol (Invitrogen) 16 h p.i. The presence of viral RNA in both infected and uninfected control cells was tested by PCR using non-structural protein 3-specific (nsP3) primers. The quality of the samples was assessed by NanoDrop ND-1000 spectrophotometer (Labtech). A total of 30 µg of RNA pooled from 3 separate infected or uninfected wells were sent for 454 pyrosequencing (Roche FLX)(Genepool, Edinburgh). 50 Mb were sequenced per sample.

The sequences were retrieved in FASTA format and contigs were assembled *de novo*. Contig coverage was calculated as a form of transcript quantification. Contigs from both samples were aligned to the *Ae. aegypti* genome (GenBank ref. AAGE00000000). The confidence value used was 1e-05. The infected sample also had the contigs aligned against SFV genome (GenBank ref. X04129) with an e-value of 1e-01. This lower confidence value was used since a 1e-05 value only showed 9 hits. Random sampling of 15 these contigs was analysed through Basic Alignment Search Tool (BLAST). Each of the sampled contigs aligned to the SFV genome showing that the stringiness was sufficient to include SFV transcripts without obtaining any *Ae. aegypti* transcripts. The contig coverage was also provided, giving some quantification to the sequencing. The contig coverage was calculated using the following formula:

$$\text{coverage of (x) contig} = \frac{\text{Number of repeats of (x) transcript x lenght of average contig (250)}}{\text{Lenght of (x) contig}}$$

Phenoloxidase activity assay

Conditioned cell culture medium from *Ae. albopictus*-derived U4.4 mosquito cells was harvested 48 h post-cell seeding (4x10^6 cells in a 75cm2 flask) and was centrifuged at 2000 rpm for 5 min in order to eliminate residual cells. Approximately 5 µl of pelleted *E. coli*

JM109 culture (New England Biolabs) or 3.5 x 10^7 PFU of SFV were added to 1 ml of cell culture supernatant and incubated for 10 min at room temperature. The mixture was then centrifuged at 3000 x g for 10 min at 4˚C in order to remove the bacteria. Phenoloxidase (PO) activity assays were carried in 96-well plates with 20 µl of cell culture medium added to 100 ul of substrate (50 mM Sodium Phosphate buffer (pH 6.5) containing 2 mM dopamine (Sigma-Aldrich)). PO activity was monitored by measuring absorbance at 490 nm using a plate reader (Dynatech MR5000) over a period of 30 min. It should be noted that this assay predominantly detects dopachrome and/or dopaminechrome rather than melanin itself. One unit of PO activity was defined as $\Delta A490 = 0.001$ after 30 min, as previously described (Beck and Strand, 2007). Each experimental condition was measured in 10 independent reactions per experiment.

Cell staining for PPO

Intracellular PO activity was detected by fixing U4.4 cells in wells with glacial methanol (100%)(Sigma Aldrich). Glacial methanol in addition to fixing the cells also activates PPO into PO. Cells were then rinsed with PBS and incubated for 1 h in 50 mM Sodium Phosphate buffer (pH 6.5) containing 2 mM dopamine (Sigma-Aldrich).

Statistics

Statistical analysis in chapters 4 and 5 were done with the assitance of Dr. Margo Chase-Topping, from the Centre for Immunity, Infection and Evolution, Ashworth Laboratories, Kings Buildings, University of Edinburgh. Statistical analysis in the rest of the chapters was done using Prism 5 (GraphPad Software) Data with 2 groups were analysed using either t-test, in luciferase assays, or Mann Whitney tests, in the case of PO activity assays. Data with more than 2 groups was analysed using General Linear Models (GLM). Survival analysis curves were tested using Kaplan-Meier estimator and the log-rank test. Where appropriate, multiple comparisons were performed and the Bonferroni correction was applied. Kaplan-Meier tests and GLM were conducted using SAS v9.1.3 (SAS Institute Inc., Cary, NC, USA). Analysis with $p<0.05$ values would be reported as exhibiting formal statistical significance.

Chapter 3. The transcriptome of SFV4 infected U4.4 cells

INTRODUCTION

In recent years, transcriptome analyses have become a popular tool to identify differentially regulated genes. Most research in mosquitoes however, focuses on infection of *Ae. aegypti* mosquitoes using gene arrays (Bonizzoni et al., 2012; Colpitts et al., 2011; Price et al., 2011; Sim, Ramirez, and Dimopoulos, 2012). The practical reason is that, unlike *Aedes albopictus*, the *Aedes aegypti* genome has been sequenced and annotated (Nene et al., 2007). Transcriptome analysis is likely to become more popular for studies in mosquitoes in the future. Contig assembly and aligments are a considerably easier task when a reference genome is available. Annotated genomes enable the use of technologies like RNA pyrosequencing or microarrays which give more detailed quantification and analysis of gene regulation. Several arbovirus vector genomes, such as insects and ticks, have been annotated, at least in part (see table 3.1), however, important vectors such as *Ae. albopictus* still require a reference genome. There has been a previous investigation into the transcriptome of *Ae. albopictus* derived C6/36 cells challenged with bacteria and there are many similarities between that study and this one (Dixit, Patole, and Shouche, 2011). Comparisons between those transcriptomes already published will be further analysed in the discussion.

Organism	Strain	Assembly Version	Gene build
An. gambiae	PEST, M, S	AgamP3, Feb 2006 AgamM1, Oct 2010 AgamS1, Oct 2010	AgamP3.6, Dec 2010
Ae. aegypti	Liverpool LVP	AaegL1, Mar 2006	AegL1.3, April 2012
C. quinquefasciatus	Johannesburg (JHB)	CpipJ1	CpipJ1.3, April 2012
I. scapularis	WIKEL	IscaW1	IscaW1.2, April 2012

Table 3.1. Sequence data available to this date for vector organisms. Major vector arthropods, except for Ae. albopictus, have their genome sequenced and assembled, however notation and idenfication of gene function are still on progress. (vectorbase.org)

Here we use the Roche 454 platform to sequence and assemble the transcriptome of *Ae. albopictus* derived U4.4 cells infected or mock-infected with SFV. This will also generate some valuable information about this cell line and mosquito species.

Sequencing platforms: Roche's 454 vs. Illumina's Solexa

RNA sequencing technologies advance as fast as digital technologies. Both Roche 454 and Illumina Solexa technologies were recently available at the time of the study. Illumina Solexa produces more readings and a deeper sequencing than Roche 454, but shorter reads (~250 bp). Solexa sequencing technology is based on reversible dye-terminators. DNA molecules are first attached to primers on a slide, and then they are amplified so that local clonal colonies are formed. This process is known as bridge amplification. Four types of reversible terminator bases are added, and non-incorporated nucleotides are discarded. DNA in these amplifications can only be extended one nucleotide at the time. A camera takes images of the fluorescently labelled nucleotides, then the dye, along with the terminal 3' blocker, is chemically removed from the DNA, allowing it to proceed to the next cycle (Mardis, 2008b). However, the 454 produced longer readings than Solexa sequencing that could be easily assembled against a reference genome if the original species genome is not available, such as it was in this case.

454 Life sciences developed a version of pyrosequencing, which was then was acquired by Roche Diagnostics. A workflow diagram of 454 pyrosequencing can be seen in figure 3.1. In 454 sequencing, DNA is amplified inside an aqueous buffer droplet in an oil solution, with each droplet containing a single DNA template attached to a single primer-coated bead, which then forms a clonal colony. The sequencing apparatus, known as a picotitre plate (PTP), contains many wells each containing a single bead and sequencing enzymes. Pyrosequencing uses luciferase to generate light for detection of the individual nucleotides added to the nascent DNA. The combined data is used to generate sequence read-outs (Margulies et al., 2005). This technology provides intermediate read length and not achieved with Solexa sequencing.

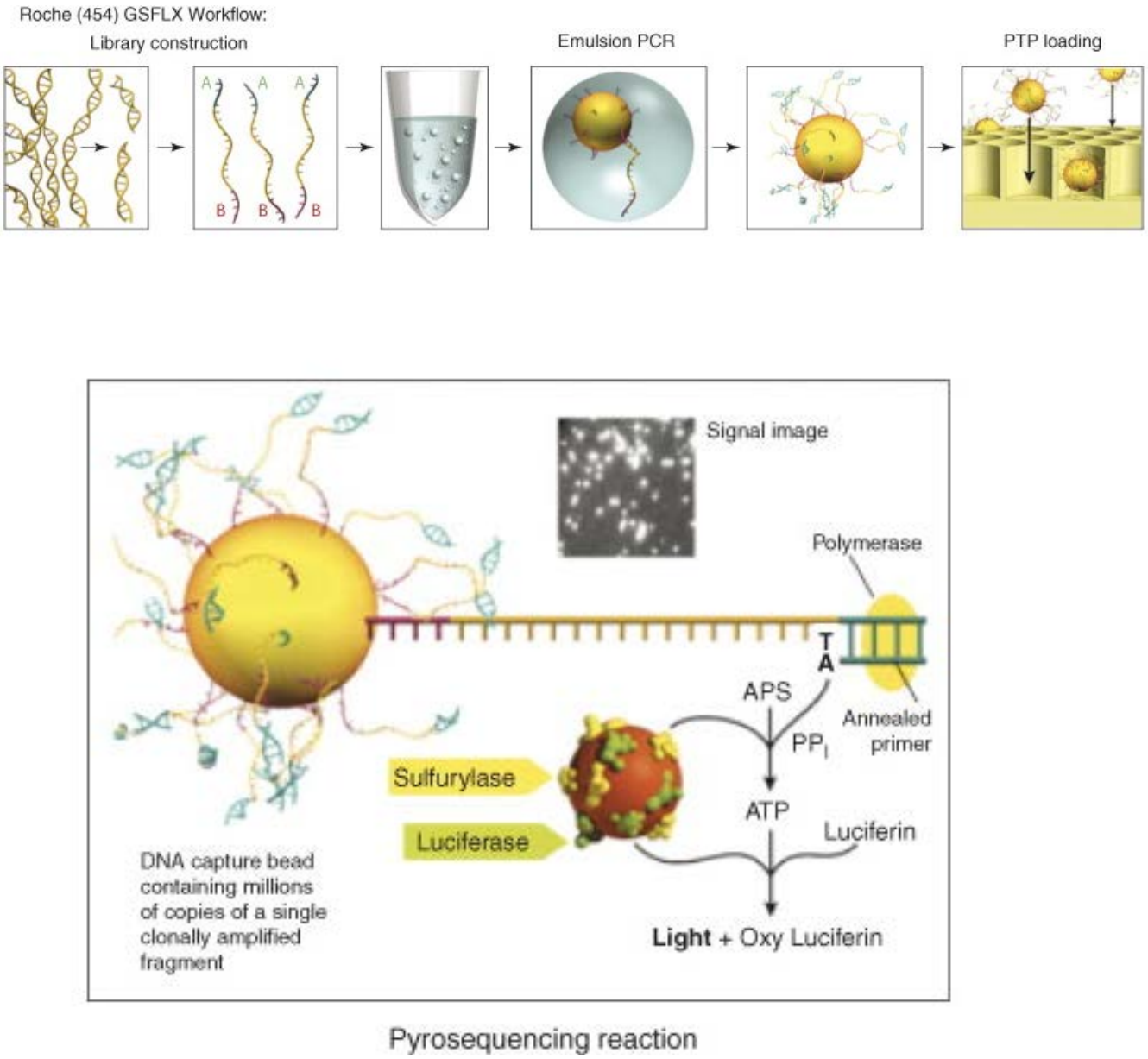

Figure 3.1. Roche 454 sequencing workflow. During library construction 454-specific adapters are bound to the DNA fragments. This couples the amplification beads with DNA in an emulsion PCR to amplify fragments before sequencing. The beads are loaded into the diminute wells which cover the picotiter plate. The bottom panel illustrates the pyrosequencing reaction that occurs on nucleotide incorporation to report sequencing by synthesis. From (Mardis, 2008a).

Objectives

- Generation of a transcriptome database for the study of *Ae. albopictus* functional genetics, due to the limited genetic information currently available for this mosquito species. Once the data base has been generated, the sequences of transcripts can be, for example, used to for a gene silencing experiment.
- Identification of transcripts differentially regulated in SFV4 infection of U4.4 cells in order to obtain an overview of immune responses in this cell line.

454 pyrosequencing and contig assembly. Experimental set up.

A six-well plate with 6.5 x 10^5 U4.4 cells per well was infected with a multiplicity of infection of 10 PFU of SFV4. A similar plate was mock-infected. Total RNA from infected and uninfected U4.4 cells was isolated using Trizol (Invitrogen) 16 hours post-infection. This time point was chosen because it near is the transition point between the acute phase and the persistent phase of viral infection in U4.4 mosquito cells. The presence of viral RNA in both infected and uninfected control cells was tested by PCR using SFV non-structural protein 3-specific (nsP3) primers. The quality of both samples was assessed by NanoDrop ND-1000 spectrophotometer (Labtech). A total of 30 µg of RNA from 3 separate infected or uninfected wells were pulled together into one sample for each condition (infected and uninfected) and sent for 454 pyrosequencing (Roche FLX) (Genepool, Edinburgh). 50 Mb were sequenced per each of the two samples.

The sequences were retrieved in FASTA format and contigs were assembled *de novo* using an algorithm for self aligment. This step was done by the bioinformatics team who provided the sequencing service at Genepool, University of Edinburgh. The low value cut off value for the reading length is 90 bp so no siRNAs or viRNAs were analysed in this study. Contigs from both samples where then aligned to the *Ae. aegypti* genome (GenBank ref. AAGE00000000) in order to identify cellular genes. A contig (from contiguous) is a set of overlapping DNA segments that together represent a consensus region of DNA. When using 454 sequencing not all the transcripts are complete, mostly they are fragmented. Moreover, 454 sequencing occurs indiscriminately from either the 3' end or the 5' end of the transcript. Therefore we cannot assume that each fragment of transcript does equal a whole transcript. Contig coverage takes into account the transcript length as well as the number of repeats it appears. It represents the number of nucleotide bases belonging to each gene which has been sequenced. In *de novo* sequencing projects, such as this one, a contig refers to overlapping sequence data (reads) (Gregory, 2005). The confidence cut-off value used for the aligment was 1e-05 ensuring that there was a significant probability that homologue genes were notated. The infected sample also had the contigs aligned against SFV genome (GenBank ref. X04129) with a cut-off e-value of 1e-01. This lower confidence value was used since a 1e-05 value showed only 9 hits. A random sampling of 15 of these contigs were analysed using Basic Alignment Search Tool (BLAST). Each of the sampled contigs aligned to the SFV genome showed that the stringiness that was enough sufficient to include SFV transcripts without obtaining any *Ae. aegypti* or other species of transcripts included. The contig

coverage was also provided, giving some quantification to the sequencing and was calculated using the following formula:

$$\text{coverage of (x) contig} = \frac{\text{Number of repeats of (x) transcript x lenght of average contig}}{\text{Lenght of (x) contig}}$$

Normalisation

Normalisation between the RNA from uninfected and infected cells was attained by equalizing the contig coverage number in the 40S ribosomal protein S7. It is established that the levels of this transcript generally do not change upon cell stress or infection in mosquitoes and it has been used in several transcriptome microarray analysis (Das et al., 2010; Zou et al., 2011) based on data from (Bolstad et al., 2003).

RESULTS

Sequencing of RNA transcript and data analysis

A total of 102,689,480 bp were sequenced from the infected sample, whereas 76, 401, 262 bp were sequenced from the uninfected sample. From the infected sample 10332 contiguous reads (transcripts) were made with an average length of 301 bp per read and an average of 11.48 copies per transcript. In case of the uninfected sample, 13099 contiguous transcripts were sequenced with an average length of 292 bp and 11.88 copies per read.

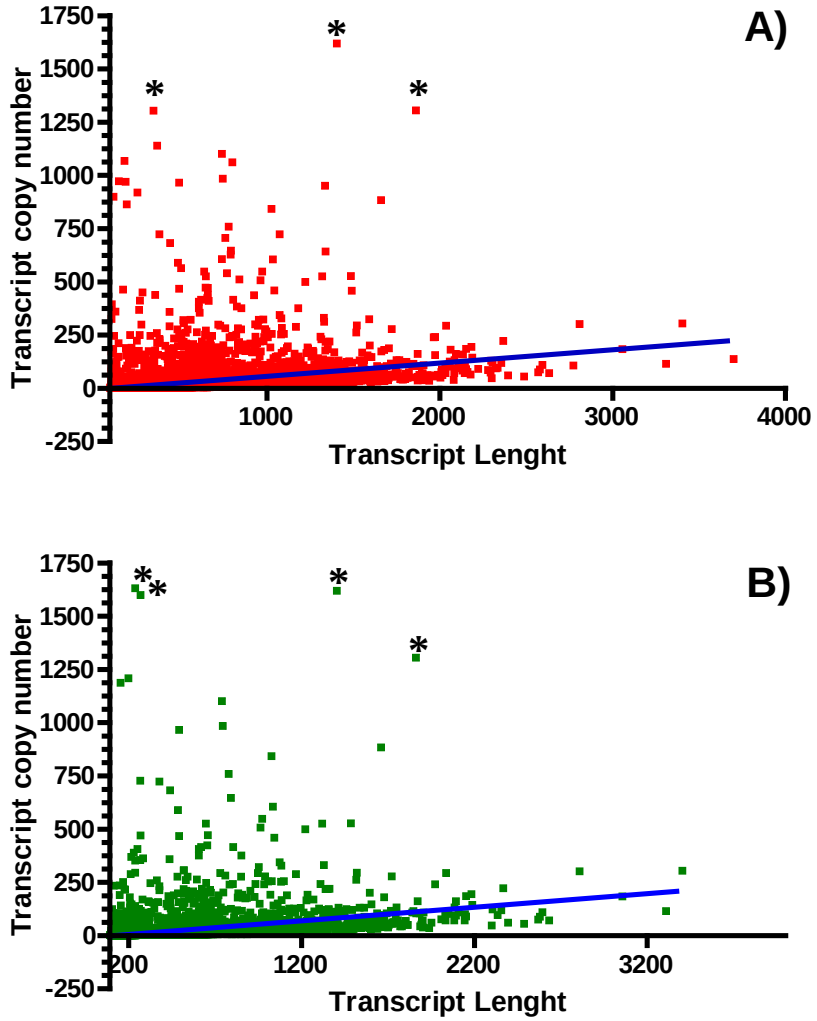

Figure 3.2. Profile of RNA transcripts. Transcripts of RNA are plotted according to length and copy number. Infected cells (A) and uninfected cells (B) distribution. Highly abundant transcripts, above 1250 copies, are marked (*).

There is no difference between the length and transcript number distribution between the infected and the uninfected samples. However, in both samples there is a tendency towards a high abundance of longer transcripts (linear regression infected: r^2=0.01219 linear regression uninfected: r^2=0.01127, analysis done in Prism5).

Origin of RNA transcripts in U4.4 cells

After calculating the proportion of SFV or host cell transcripts from the infected sample it shows that most of the RNA transcripts sequenced derived for the host cell. The number of viral transcripts sequenced in relation to the total number of RNA transcripts is very small (Fig.3.2). It is interesting to notice that, 1312 transcripts from the infected sample aligned to the SFV sequence. The total infected sample coverage was 79400. Thus only 1.65% of the total RNA isolated from the infected cells belonged to SFV. The alignment of these transcripts to the viral genomes, as well as the possible rise of mutations or variants in the culture was not investigated in this study.

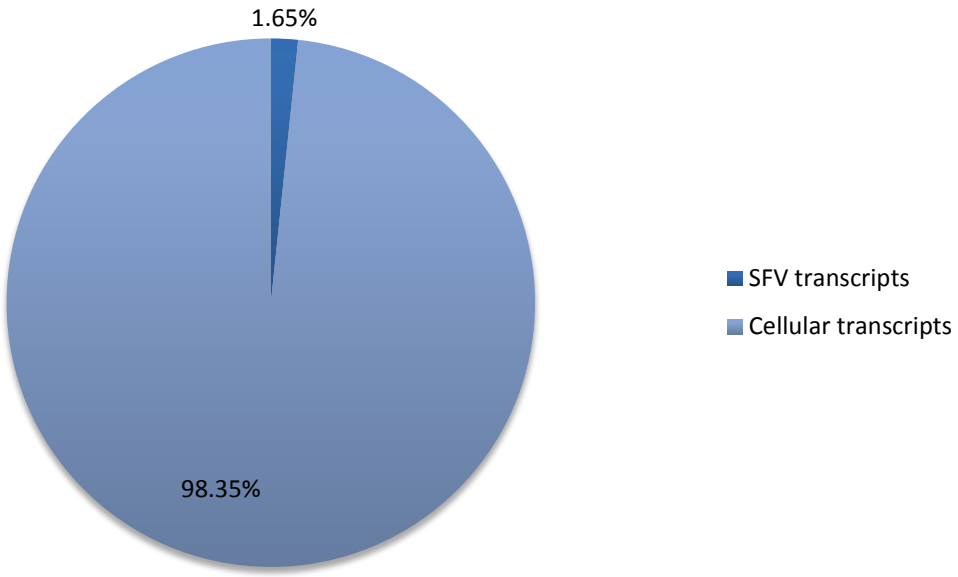

Figure 3.3. Percentage of origin of total RNA sequenced transcripts from infected U4.4 cells. Of a total of 79400 transcripts sequenced, 1312 (1.65%) were of viral origin whereas 78088 (98.35%) were of cellular origin.

Identification of transcripts in U4.4 cells

RNA transcripts were assembled into genes and aligned to the *Ae. aegypti* genome for annotation. The total number of unique *Ae. albopictus* transcripts identified after combining both samples was 2874, out of which 222 were unique to the uninfected sample; 399 genes were unique to the infected sample and 2253 genes were present in both infected and uninfected.

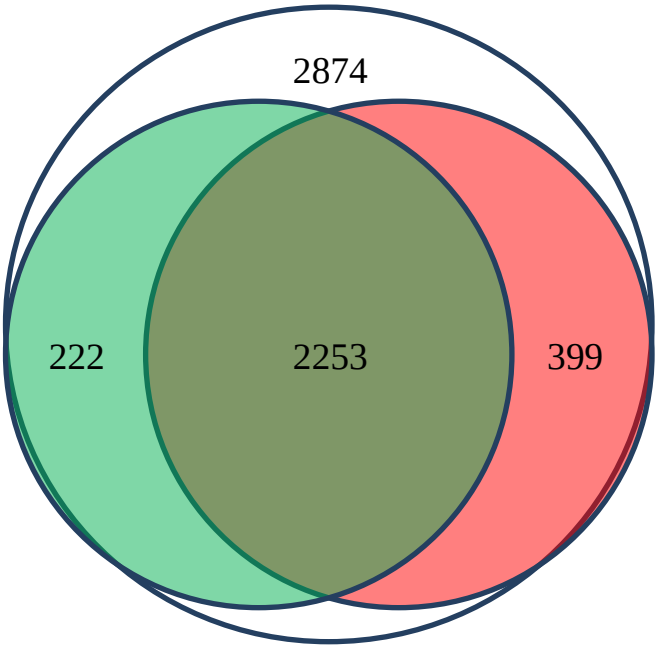

Figure 3.4 Number of transcripts identified in the sequencing. The total number of unique transcripts identified are in the large white circle; present uniquely in the uninfected sample are in green and genes unique to the infected sample in red. Genes present in both infected and uninfected appear in the convergent zone.

Not all the transcripts mapped to annotated genes in the *Ae. aegypti* genome. A total of 651 (22.6%) transcripts coded for unknown hypothetical genes; 7 (3.11%) were present only in the uninfected cells and 644 (28.58%) present in both infected and uninfected cells. Those are very high percentages, which imply that, although the *Ae. aegypti* mosquito genome has been sequenced, there is still a great proportion of genes that need to be annotated.

Differential gene expression during SFV infection of U4.4 cells

The following analysis takes into account those transcripts present in both infected and uninfected cells. We decided the threshold to classify a gene as 'up-regulated' or 'down-regulated' was a 2 fold change in differential gene expression which, in this case, is represented as contig coverage. It was found that 2091 genes were not differentially expressed; 99 genes were down-regulated and 32 genes were up-regulated when comparing genes present in both samples.

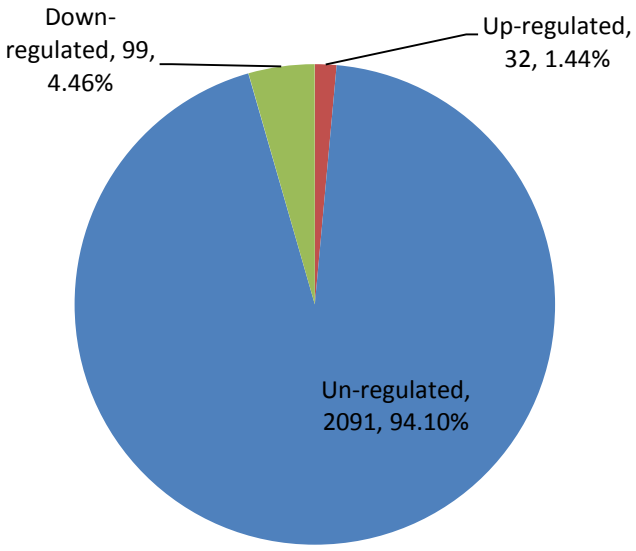

Fig. 3.5. Differential gene expression during SFV infection of U4.4 cells. Down-regulated genes depicted in green, and up-regulated genes in red. The individual gene numbers are followed by the percentage. Transcripts are not present in similar numbers between infected and non-infected cells indicated in blue.

SFV infection of U4.4 cells does not lead to strong differential expression of host genes. A 94% of the genes did not show any differential expression. It has to be noted however, that SFV infection results almost three times as many down-regulated genes than up-regulated genes. In SFV4 infection of U4.4 a mild shut down of gene expression was also observed, thus inhibiting immune signalling. Both up- and down-regulated differentially expressed genes, were selected for further analysis (Fragkoudis et al., 2008c).

Analysis of up-regulated transcripts

Transcripts with at least a 2 fold difference up-regulated in contig coverage were considered for further analysis. A total of 32 genes fell into this criterion. These genes were classified ontologically (see table 3.2). A brief description of several functional classes will be provided. A quarter of the up-regulated genes are of unknown function when aligned and annotated, therefore they were not classified.

***Ae. aegypti* gene homologue**	**Annotation**	**Fold upregulation**	**Ontology**
AAEL003383-RA	actin	57.09138998	ST
AAEL004172-RA	tubulin alpha chain	11.73217832	ST
AAEL014414-RA	dead box ATP-dependent rna helicase	10.7202167	NAP, DV
AAEL011206-RA	aminoacylase, putative	7.85771773	MT
AAEL013310-RA	Mytosis associated protein	7.715596987	CC
AAEL013656-RA	BM-40 SPARC precursor	4.442301208	DV, CS, CC
AAEL007967-RA	Hypothetical serpin	4.236093292	SIG, IM
AAEL001952-RA	28 kDa heat- and acid-stable phosphoprotein (PDGF-associated protein), putative	4.228247768	UK
AAEL000951-RA	elongation factor 1-beta2	4.113695127	NAP
AAEL003336-RA	conserved hypothetical protein	3.959790223	UK
AAEL003610-RA	serine protease	3.730662808	SIG
AAEL006946-RA	Chaperonin	3.414740273	MT, IM, CS
AAEL000055-RA	conserved hypothetical protein	3.307766339	UK
AAEL003294-RA	fibrinogen and fibronectin	3.266488925	IM
AAEL000270-RA	26S proteasome regulatory subunit 7, psd7	3.070958411	MT, IM
AAEL010906-RA	conserved hypothetical protein	2.929196358	UK
AAEL010558-RA	conserved hypothetical protein	2.863533033	UK
AAEL015458-RA	Transferrin	2.759020551	MT, CS
AAEL015143-RA	glycine rich RNA binding protein, putative	2.721155225	NAP
AAEL007006-RA	serine protease	2.641147228	SG
AAEL008192-RA	40S ribosomal protein S3	2.546307913	MT
AAEL012836-RA	cytochrome B561	2.503256607	CS
AAEL011803-RA	prohibitin, putative	2.503055097	ST
AAEL004500-RA	eukaryotic translation elongation factor	2.493390207	NAP
AAEL000147-RA	single-stranded DNA binding protein, putative	2.240509563	NAP, IM, CS
AAEL002334-RA	eukaryotic translation initiation factor 3 subunit	2.22969323	NAP
AAEL012095-RA	26S protease regulatory subunit	2.22168645	MT, CS

AAEL012523-RA	transcription factor TFIIH-subunit, putative	2.098220899	NAP
AAEL007702-RA	Chaperonin	2.043692212	MT, IM, CS
AAEL006952-RA	conserved hypothetical protein	2.027205499	UK
AAEL010954-RA	conserved hypothetical protein	2.002891026	UK
AAEL008802-RA	conserved hypothetical protein	2.00082568	UK

Table 3.2 Up-regulated genes in SFV infection of U4.4 cells. Genes were annotated after aligment to the *Ae. aegypti* genome classified by fold differences in expression and ontology: UK (unknown), NAP (Nucleic Acid processing), MT (metabolism), IM (immunity), CS (cell stress), ST (structure), SG (Signalling), DV (development). Genes not annotated by the *Ae.aegypti* genome are stated (*D. melanogaster, C. pipiens, A. gambiae*).

Cell structure

Actin and tubulin, two proteins involved in cell trafficking and structure, are the most highly up-regulated molecules in this study. There are several mechanisms in which these two proteins could interact with SFV. It has been shown that in Bunyamwera virus infection of *Ae. albopictus* derived C6/36 the virus modulates the cellular actin-matrix scaffold to facilitate replication in virus factories as well as the exportation of enveloped virions. In this process the virus also changes the morphology of the cell creating pseudopodia by actin scaffold remodelling (López-Montero and Risco, 2011). In U4.4 cells however, changes in cell morphology upon SFV infection where not detected by video-microscopy (Ricky Siu PhD thesis, 2010, University of Edinburgh). Another study, this time with DENV-2, suggests that actin and tubulin, also highly up-regulated in DENV-2 infection, might act as receptors to help flavivirus entry (Paingankar, Gokhale, and Deobagkar, 2010). Prohibitin is an evolutionary conserved multifunctional protein with ubiquitous expression, however, its molecular roles are largely unknown. It has been found in drosophila, mosquitoes and silkworm (Lv et al., 2012; Paingankar, Gokhale, and Deobagkar, 2010). It is suggested that prohibitin is utilised by DENV-2 as a receptor to gain entry (Kuadkitkan et al., 2010; Paingankar, Gokhale, and Deobagkar, 2010). It is tempting to speculate that SFV utilises these structural proteins in the same way as DENV-2, however further studies should be done to reach this conclusion.

Nucleic acid processing

Genes in this category are involved in nucleic acid replication, transcription as well as nucleic acid processing. It is unknown if any of these genes have any immunological function however this group is differentially regulated in several transcriptome studies (Bartholomay et al., 2004; Colpitts et al., 2011; Sim, Ramirez, and Dimopoulos, 2012). The function of the Deadbox atp-dependent RNA helicase (AAEL014414-RA) is not known, however, upregulation of this molecule has also been found in DENV infected *Ae. aegypti* derived Aag2 cells (Sim and Dimopoulos, 2010). It has been recently shown that Drosophila Dcr-2, a DExD/H-box helicase, is capable of sensing viral dsRNA aind inducing the production of a putative antiviral effector molecule (Deddouche et al., 2008). It is also noteworthy mentioning that, in this study there is not differential expression of Dcr-2 or other RNAi pathway component.

Immunity

Several serine proteases, fibrinogen and fibronectin transcripts are up-regulated upon SFV infection of U4.4 cells. Fibrinogen and fibronectin have also been found up-regulated in DENV infected Aag2 cells (Sim and Dimopoulos, 2010). Although nothing is known about the potential role of serine proteases in arbovirus immunity, their up-regulation suggests these cells are able to mount other immune responses in addition to the established antiviral IMD, Jack-STAT and RNAi pathways. Serine proteases and serine proteases inhibitors (serpins) control several immunity related pathways in insects, such as melanisation (Cerenius and Söderhäll, 2004; Felföldi et al., 2011). These pathways are known to activate immune responses against fungi, bacteria and parasites in insects; however their role in viral infection has not been investigated. Further research into this aspect will be shown in following chapters of this thesis.

Members of the fibrinogen family are evolutionary conserved and contain fibrinogen-immunolecting domains which are also found in mammalian ficolins. This family of genes is present in the order Diptera and, interestingly, it is more abundant in mosquitoes families than in *Drosophila* (Wang, Zhao, and Christensen, 2005) Fibrinogens and fibronectings have the ability once secreted, to form multimers in the cell supernatant or, in the case of whole insects, in the haemolymph. These multimers can occur in numerous combinations and possess different binding specificities towards microbial products, including parasites and bacteria, by acting as PAMP receptors and/or trapping the microbe (Cirimotich et al., 2010;

Dixit et al., 2008; Wang et al., 2004). However, to the date, no investigation has been done to assess the role of these molecules in viral immunity in insects.

Cell stress and apoptosis

Chaperonins, proteasomes, SPARC (Secreted Protein, Acidic and Rich in Cystein), transferrin and cytochrome are up-regulated as a result of stress response induction. In mammalian cells, the build-up of SFV capsid protein and DENV proteins are known to induce ER stress which evolves into proteasome activation and apoptosis (Barry et al., 2010; Doolittle and Gomez, 2011). In insect cells, baculovirus, DENV and ONNV infection is known to induce a proteasome and heatshock protein, HsP70-90, activity upon infection (Carinhas et al., 2011; Lyupina et al., 2011; Sim and Dimopoulos, 2010; Waldock, Olson, and Christophides, 2012). Upregulation of cytochrome was also found in flavivirus infection of mosquitoes (Colpitts et al., 2011). However, to the date, there is no study that characterises the unfolded protein response in insect cells.

The role of SPARC in drosophila cells has been well characterised. During development and stress situations, ‘winner cells’ eliminate ‘losing’ neighbouring cells by inducing apoptosis. Winner cells then proliferate to even the cell numbers. SPARC is secreted by the ‘loser’ cells inhibiting apoptosis (see Figure 3.5) (Portela et al., 2010). It is unknown if the up-regulation of this gene is driven by the cell upon infection or by the virus. It can only be speculated how the virus would benefit from keeping infected cells from being eliminated by continuous virus production from infected ‘losing cells’.

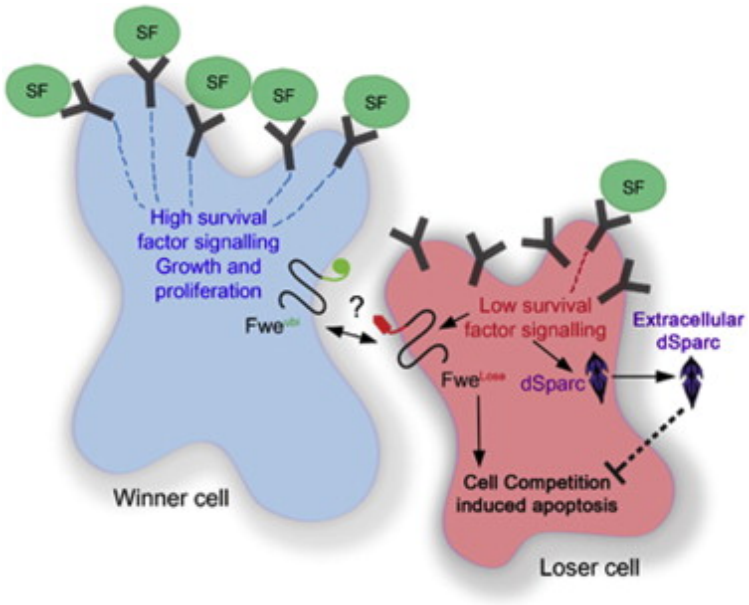

Fig 3.6 The role of SPARC in cell elimination in *D. melanogaster*. 'Loser' cells with low survival factor signalling (SF) inhibit apoptosis by production of SPARC. Adapted from (Portela et al., 2010).

Down-regulated transcripts

As with the up-regulated genes, in this study we only consider transcripts with a fold difference of at least two fold contig coverage (expression) between the infected and the uninfected sample RNA.. A total of 99 genes fell into this criterion. These genes were classified ontologically (see table 3.3). A brief description of several functional classes will be provided. A total of 21 down-regulated genes have unknown function when aligned and annotated, therefore they were not classified ontologically.

gene	notation	Fold up/down regulation	Ontology
AAEL011634-RA	fibrinogen and fibronectin	-37.24816	IM
AAEL000186-RA	conserved hypothetical protein	-23.99085	UK
AAEL011302-RD	annexin	-18.45973	MT, IM
AAEL013937-RA	serine protease inhibitor, serpin	-16.76929	SG, IM
AAEL013731-RA	kynurenine formamidase	-15.65555	MT
AAEL014548-RA	peroxiredoxins, prx-1, prx-2, prx-3	-14.09325	CS
AAEL004859-RA	atp-dependent rna helicase	-12.13185	NAP
AAEL014944-RA	cytochrome c oxidase polypeptide	-10.86334	CS
AAEL004381-RA	capicua protein	-8.556937	NAP
AAEL006438-RA	dolichyl glycosyltransferase	-7.662562	MT
AAEL002851-RA	tubulin beta chain	-7.595396	ST
AAEL013236-RA	proteasome subunit beta type 5,8	-6.431762	CS
AAEL011616-RA	serine protease, putative	-6.199566	SG, IM
AAEL005084-RA	tubulin beta chain	-5.903127	ST
AAEL007383-RD	secreted ferritin G subunit precursor, putative	-5.732737	CS, IM
AAEL009078-RA	nadh-ubiquinone oxidoreductase sgdh subunit	-5.219013	CS
AAEL008920-RA	anopheles stephensi ubiquitin, putative	-5.201915	MT, CS
AAEL013045-RA	exosome complex exonuclease RRP41, putative	-4.872546	NAP
AAEL007368-RA	hypothetical protein	-4.79003	UK
AAEL007451-RA	wd-repeat protein	-4.698928	SG, NAP, ST
AAEL004276-RA	C. pi - C10 protein	-4.600326	IM
AAEL001164-RA	NADH:ubiquinone dehydrogenase, putative	-4.326879	MT
AAEL012812-RA	exosome complex exonuclease RRP41, putative	-4.19555	NAP
AAEL004740-RA	35 kDa GTP-binding protein, putative	-4.186419	UK
AAEL002368-RA	C. pi ubiquitin/ribosomal protein S27a	-3.995776	MT
AAEL001547-RA	hypothetical protein	-3.995776	UK

AAEL004060-RA	C. ele Putative ATP synthase epsilon chain, mitochondrial	-3.949477	UK
AAEL010170-RA	ras-related protein Rab-8A, putative	-3.916731	SG, IM
AAEL000508-RA	fibrinogen and fibronectin	-3.772682	IM
AAEL010318-RA	polyadenylate-binding protein	-3.646749	NAP
AAEL011905-RA	myosin i	-3.577896	ST
AAEL012875-RA	snare protein sec22	-3.558144	MT
AAEL004070-RA	hypothetical protein	-3.445366	UK
AAEL009140-RA	hypothetical protein	-3.437438	UK
AAEL008153-RA	C. pi- arrestin domain-containing protein 2	-3.424532	UK
AAEL000804-RA	conserved hypothetical protein	-3.321268	UK
AAEL002825-RA	NADH:ubiquinone dehydrogenase, putative	-3.307712	MT
AAEL008403-RA	C. pi- 26S proteasome non-ATPase regulatory subunit 10	-3.284695	CS
AAEL007514-RA	oviductin	-3.215837	SG, IM
AAEL002797-RA	conserved hypothetical protein	-3.172963	UK
AAEL007820-RA	Human LSM12 homolog	-3.160261	NAP
AAEL011773-RA	calreticulin	-3.14544	CS, IM
AAEL005678-RA	UDP-galactose transporter	-3.097463	MT
AAEL008741-RA	importin (ran-binding protein)	-3.060561	NAP
AAEL013942-RA	aminoadipate-semialdehyde dehydrogenase	-3.050334	MT
AAEL004823-RA	superoxide dismutase [mn]	-2.99446	CS
AAEL002183-RA	oligosaccharyl transferase, subunit, putative	-2.949713	MT
AAEL005636-RA	eukaryotic translation initiation factor 2b, epsilon subunit	-2.947442	NAP
AAEL011293-RA	vitamin-K-epoxide reductase (warfarin-sensitive), putative	-2.92007	CS
AAEL009667-RA	conserved hypothetical protein	-2.917119	UK
AAEL001194-RA	fatty acid synthase	-2.839667	MT
AAEL000138-RA	NADH dehydrogenase, putative	-2.78521	MT
AAEL012862-RA	hypothetical protein	-2.768201	UK
AAEL003749-RA	Drosop. -Transcription elongation factor 1 homolog	-2.687762	NAP
AAEL003344-RA	metaxin	-2.653099	CS
AAEL012679-RA	Juvenile hormone-inducible protein, putative	-2.635014	CS
AAEL010361-RA	rer1 protein	-2.609322	ST
AAEL013613-RA	pyruvate dehydrogenase	-2.504608	MT
AAEL004282-RA	protein-(glutamine-N5) methyl transferase, putative	-2.472741	MT
AAEL011155-RA	Human Required for meiotic nuclear division	-2.472178	CC

	protein 1 homolog.		
AAEL009474-RA	peptidoglycan recognition protein-lc isoform	-2.471118	IM
AAEL004997-RA	U3 small nucleolar ribonucleoprotein protein imp4	-2.465714	NAP
AAEL002427-RA	transcription factor IIIB 90 kDa subunit (TFIIIB90)	-2.457541	NAP
AAEL005638-RA	C. pi- 60S ribosomal protein L7	-2.412454	MT
AAEL006727-RA	multisynthetase complex, auxiliary protein, p38, putative	-2.386908	CS
AAEL006458-RA	alcohol dehydrogenase	-2.380221	MT, CS
AAEL004808-RA	Dros - double-stranded RNA binding	-2.358896	UK
AAEL012161-RA	Dros- Aldehyde dehydrogenase type III	-2.34245	MT
AAEL009305-RA	numb-associated kinase	-2.341348	SG
AAEL007375-RA	pyruvate dehydrogenase	-2.337085	MT
AAEL013260-RA	alpha methylacyl-coa racemase	-2.326412	MT,
AAEL002304-RA	porphobilinogen synthase	-2.298104	MT
AAEL003695-RA	conserved hypothetical protein	-2.283035	UK
AAEL010227-RA	dolichol-phosphate mannosyltransferase	-2.282551	MT
AAEL005146-RA	conserved hypothetical protein	-2.253033	UK
AAEL009373-RA	n-acetyltransferase	-2.237042	MT
AAEL010501-RA	zinc finger protein	-2.229736	UK
AAEL012313-RA	charged multivesicular body protein 5	-2.21963	ST, MT
AAEL006395-RA	hypothetical protein	-2.204883	UK
AAEL007980-RA	hypothetical protein	-2.201734	UK
AAEL001077-RA	conserved hypothetical protein	-2.180407	UK
AAEL002843-RA	conserved hypothetical protein	-2.167397	UK
AAEL008738-RA	dead box atp-dependent rna helicase	-2.165336	NAP
AAEL012538-RA	conserved hypothetical protein	-2.163989	UK
AAEL005880-RA	conserved hypothetical protein	-2.161065	UK
AAEL009227-RA	kinesin heavy chain	-2.150995	CS
AAEL005829-RA	conserved hypothetical protein	-2.124817	UK
AAEL013890-RA	26S proteasome non-atpase regulatory subunit	-2.120998	CS
AAEL003634-RA	Hsp70-interacting protein, putative	-2.090667	CS
AAEL007822-RA	ubiquitin-conjugating enzyme E2 g	-2.080365	CS
AAEL010430-RA	ras-related protein, putative	-2.077866	SG, IM
AAEL009483-RA	conserved hypothetical protein	-2.075132	UK
AAEL007300-RA	syntaxin binding protein-1,2,3	-2.053778	NAP, IM
AAEL009671-RA	snrnp sm protein	-2.050406	NAP
AAEL011611-RA	serine protease, putative	-2.042331	SG, IM
AAEL001872-RA	voltage-dependent anion-selective channel	-2.041745	ST, MT

AAEL006764-RA	glutathione transferase D10, putative	-2.035482	CS, IM
AAEL013410-RA	conserved hypothetical protein	-2.032396	UK
AAEL007009-RA	conserved hypothetical protein	-2.017594	UK

Table 3.3. Down-regulated genes in SFV infection of U4.4 cells. Genes were annotated after aligment to the *Ae. aegypti* genome classified by fold difference of expression and ontology: UK (unknown), NAP (Nucleic acid processing), MT (metabolism), IM (immunity), CS (cell stress), ST (structure), SG (Signalling), DV (development). Genes not annotated by the *Ae. aegypti* genome are stated (*D. melanogaster, C. pipiens, A. gambiae*).

Immunity

Fibrinogen and fibronectin transcripts are down-regulated. This finding contradicts other data in this study, which suggested that fibrinogen and fibronectin were up-regulated (table 3.2). However, further investigation showed that these three genes, AAEL003294 (up-regulated), AAEL011634 (down-regulated) and AAEL000508 (down-regulated), share no homology with each other.

Annexins are members of a protein family characterized by the presence of four to eight annexin repeats. Their function is variable and they have been shown to play a role in processes such as apoptosis, differentiation, membrane fusion as well as endo- exocytosis and immunity. In *An. gambiae* and D. *melanogaster*, they bind to *Plasmodium berghei* ookinetes (Kotsyfakis et al., 2005). More importantly, they have also been implicated in the receptor mediated uptake of certain viruses, like hepatitis B or human cytomegalovirus (Depla, 2000). The role of annexins in arbovirus infection has not been investigated.

Ferritins are proteins involved in scavenging and storing of iron. They have been shown to play a key role in host-pathogen interactions, such as Wolbachia intracellular bacteria, which interferes with iron metabolism in insects (Kremer et al., 2009). To the date, no interactions between arboviruses and ferritin have been shown in insects.

The role of the Rab GTPase family in insects is unclear. While some Rab protein appears to be important for alphavirus endocytosis and entry (Colpitts et al., 2007), others are important in mTOR (mammalian target of rapamycin) activation and regulation (Li et al., 2010). The mTOR signalling pathway seems to have a role in DENV infection of *Ae. aegypti*, however it

is not well understood (Behura et al., 2011). Other proteins of the Rab family are responsible for exporting drosomycin (an anti-microbial) vesicles (Shandala et al., 2011).

Interestingly, Syntaxin binding and Snare proteins are also down-regulated in SFV infection. Syntaxin is involved in the anti-microbial peptide secretion pathway in *Drosophila* (Shandala et al., 2011), and the SNARE complex is associated with Syntaxin upon an external stimulus, such as infection (Jewell et al., 2011). It is possible that the down-regulation of both Rab and Syntaxin associated proteins that we see in SFV infection could be involved in activating the machinery of anti-microbial peptide vesicle export; however this hypothesis is still to be proven. All these anti-microbial studies have been done with bacteria, however; drosomycin has been shown to be part of the anti-viral immune response in insects (Luna et al., 2003; Thoetkiattikul, Beck, and Strand, 2005; Tsai et al., 2008).

Calreticulin is a chaperone protein associated with the ER. While in insects it has not been associated with virus infections, in human cells this protein inhibits replication of DENV. It has indeed been shown that calreticulin also colocalised with viral dsRNA and replication complexes of DENV (Khadka et al., 2011).

Cell stress

Regulators of active oxygen species, such as peroxiredoxins, are down-regulated during SFV infection of U4.4 cells. Peroxiredoxins are a ubiquitous family of antioxidant enzymes and seem to play a role in controlling baculovirus infection of *B. mori* (Lee et al., 2005). Other oxidative stress related proteins which also regulate active oxygen species, such as NADH-ubiquinone oxidoreductase, superoxide dismutase, vitamin-K-epoxide reductase, metaxin and glutathione transferases in general are all down-regulated.

Some proteosome related proteins, as with calreticulin, are down-regulated. Another protein known as Hsp70-interacting protein is also down-regulated. It is not known if this Hsp70-interacting protein is pro- or anti-viral, or even if it positively or negatively regulates proteasome complexes.

Other pathways

SFV infection of U4.4 cells also seems to down-regulate some genes involved in endocytosis (charged multivesicular body protein 5), fatty acid metabolism (alpha methylacyl-coa racemase) and ER integrity (rer1 protein). Lipid metabolism is extremely important for viruses which replicate in the cytoplasm and they induce extensive ultrastructural changes in infected cells. Host-derived membranes are rearranged to provide extensive platforms that help to assemble arrays of replication factories. Fatty acids also have signalling functions. In C6/36 infected with DENV fatty acid metabolism is highly altered (Perera et al., 2012). The fatty acid metabolism transcripts found in this study remain uncharacterised. Genes involved in mRNA processing and degradation (polyadenylate-binding protein, snrnp sm protein) are also down-regulated. As it has been shown for mammalian cells (Garmashova et al., 2006), and DENV infected Aag2 cells (Sim and Dimopoulos, 2010), there is a trend towards transcript downregulation in SFV infected U4.4 cells.

Genes uniquely expressed in infected or uninfected cells

Caution has to be taken when analysing genes present only in infected or uninfected cells. Fold difference expression cannot be calculated and contig coverage is the only quantitative available value. This information is still worthy of further analysis.

Transcripts only present in uninfected cells

The first striking observation in transcripts only present in unifected cells is the presence of kinesin, which is not detected in SFV infected cells. Kinesin are motor proteins in charge of transporting molecules along microtubules. How SFV or other arbovirus infection affects the number of transcripts of this protein is unknown. Other potentially interesting molecules only present in uninfected cells is a proteasome inhibitor (AAEL001422). This finding correlates with the fact that proteosome subunit transcripts were also found up-regulated in this study. SFV could induce the formation of proteasomes through an unfolded protein response (Table.3.4).

Gene	Notation	Fold up-down regulation	Notation
AAEL014084-RA	kinesin eg-5	4,575	CC
AAEL004484-RA	predicted protein	18.7	CC
AAEL011387-RA	leucine-rich repeat	12.22	CS
AAEL007176-RA	rabkinesin-6	9.57	CC, MT
AAEL001422-RA	proteasome inhibitor	8.98	CS
AAEL000884-RA	eukaryotic translation initiation factor 2 alpha kinase 1 (heme-regulated eukaryotic initiation factor eif-2-alpha kinase)	8.25	NAP
AAEL000983-RA	clathrin coat assembly protein ap19	7.86	ST
AAEL006150-RA	brca1-associated protein (brap2)	7.28	NAP
AAEL004942-RA	helicase	6.83	NAP
AAEL002866-RA	cyclin l	6.79	CC
AAEL003220-RA	rho-type gtpase activating protein	6.76	SG
AAEL001176-RA	s-adenosylmethionine decarboxylase	6.44	MT
AAEL002943-RA	che-11	6.36	NAP
AAEL008741-RA	importin (ran-binding protein)	6.25	ST
AAEL003759-RA	calcium-activated potassium channel alpha subunit	6.2	MT
AAEL009179-RA	molybdopterin biosynthesis protein	5.84	CS
AAEL003546-RA	gamma-tubulin complex component 4 (gcp-4)	5.83	ST
AAEL010717-RA	ecdysone receptor isoform-B (EcRB)	5.81	DV
AAEL002766-RA	glutamyl-tRNA(Gln) amidotransferase subunit A	5.81	NAP
AAEL010449-RA	huntingtin interacting protein	5.71	DV

AAEL012211-RA	microfibrillar-associated protein, putative	5.63	NAP
AAEL010432-RA	exocyst complex-subunit protein, 84kD-subunit, putative	5.56	DV
AAEL006840-RA	U2 small nuclear ribonucleoprotein a	5.43	NAP
AAEL011303-RA	cell division protein ftsj	5.32	CC
AAEL003522-RA	protein arginine n-methyltransferase	5.29	MT

Table 3.4. The top 25 transcripts only present in uninfected cells. Genes were annotated after aligment to the *Ae. aegypti* genome, classified by contig coverage and ontology: UK (unknown), NAP (Nucleic Acid processing), MT (metabolism), IM (immunity), CS (cell stress), ST (structure), SG (Signalling), DV (development) CC (Cell cycle).

Transcripts only present in infected cells

We can only assume that those genes only present in the transcriptome of infected cells are induced upon virus infection.

Gene	Notation	Contig coverage	Ontology
AAEL012353-RA	antifreeze protein, putative	18.52	CS
AAEL010668-RA	quinone oxidoreductase	8.38	CS
AAEL009080-RB	importin 7,	7.75	NAP
AAEL010119-RA	ER-derived vesicles protein ERV14, putative	7.29	ST
AAEL004425-RA	ctg4a	7	IM, SG
AAEL011822-RA	gamma glutamyl transpeptidases	6.49	MT
AAEL004098-RA	neuromusculin	6.49	IM
AAEL001333-RA	protein arginine n-methyltransferase 1,	6.44	MT
AAEL011063-RA	tumor endothelial marker 7 precursor	6.3	UK
AAEL002976-RA	Aspartyl beta-hydroxylase, putative	6.25	SG
AAEL005597-RA	dna helicase recq5	6.2	NAP
AAEL003246-RA	deoxyribose-phosphate aldolase	6.17	NAP
AAEL012154-RA	2,3-cyclic-nucleotide 2-phosphodiesterase	6.16	MT
AAEL001066-RA	mitochondrial ribosomal protein, L22, putative	6.16	MT
AAEL013652-RA	oxidoreductase	6	CS
AAEL003179-RA	protein arginine n-methyltransferase 1,	5.89	MT
AAEL014946-RA	protease U48 caax prenyl protease rce1	5.81	MT
AAEL012120-RA	fad oxidoreductase	5.76	CS
AAEL001580-RA	otefin, putative	5.73	ST
AAEL013820-RA	Bj1 protein, putative	5.64	NAP, DV
AAEL010610-RA	serine palmitoyltransferase i	5.63	MT
AAEL008649-RA	L-galactose dehydrogenase, putative	5.62	MT
AAEL011245-RA	deoxyhypusine synthase	5.61	IM
AAEL006039-RA	dehydrodolichyl diphosphate synthase (dedol-pp synthase)	5.51	MT
AAEL006833-RA	succinyl-CoA synthetase small subunit, putative	5.51	MT
AAEL001396-RA	mandelate racemase	5.49	ST

Table 3.5. The top 25 transcripts only present in infected cells. Genes were annotated after aligment to the *Ae. aegypti* genome classified by contig coverage and ontology: UK (unknown), NAP (Nucleic acid processing), MT (metabolism), IM (immunity), CS (cell stress), ST (structure), SG (Signalling), DV (development).

The profile of transcripts only present in the infected cell samples is interesting. There are several proteins related to the oxidative stress pathways, including several oxidoreductases. Their enzymatic role is the opposite of peroxiredoxins (down-regulated) and superoxide dismutases (down-regulated). Other transcripts only present in infected cells include molecules ER and cell vesicle trafficking, such as ERV14, serine palmitoyltransferase and

lipid storage droplets surface binding proteins. Immune related molecule transcripts present include ctg4a, also known as Toll receptor 4, and neuromusculin, which is an immunoglobulin. Deoxyhypusine synthase is an anti-parasitic molecule which is also down-regulated in infected cells, but its anti-viral properties have not been investigated (Moritz et al., 2004). However no trancripts of defensins or other traditionally induced IMD, JAK/STAT or Toll pathway were detected. This confirms previous observations that SFV does not induce these immune signalling pathways (Fragkoudis et al., 2008c).

Overall transcript profile

As shown before, there is a trend towards downregulation in SFV infected U4.4 cells. Downregulation of cellular transcripts is shown in other transcriptomic studies with DENV and ONNV (Sim and Dimopoulos, 2010; Sim, Ramirez, and Dimopoulos, 2012; Waldock, Olson, and Christophides, 2012). Metabolism, cell stress and immunity gene transcripts seem to be the most affected by this down-regulation. However, with the data provided here it is not possible to tell whether up-regulated or down-regulated genes are anti- or pro- viral or perhaps have no effect at all.

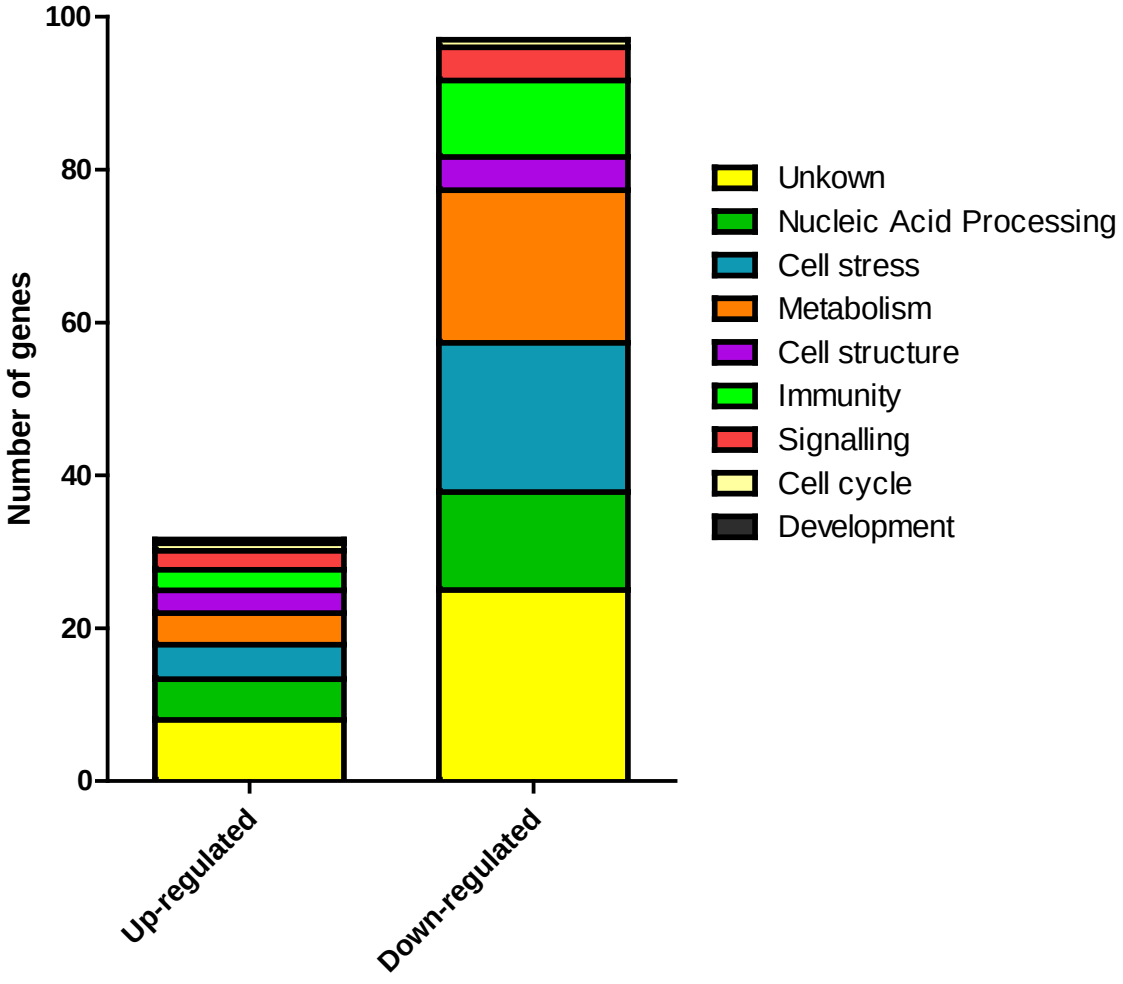

Fig.3.7. Comparison between the number of genes differentially regulated during SFV infection of U4.4 cells. Each individual gene up-regulated and down-regulated is classify by function. Gene ontology represented in colours. Ontology was manually assigned according to gene function published in the literature.

Discussion

Our hyphothesis at the beginning of the project was that SFV infection of *Ae. albopictus* derived U4.4 cells would drive differential regulation of cellular genes. In order to understand which transcripts are differentially expressed transcriptomic analysis of the total cellular RNA was carried out. The sequenced data was aligned to the *Ae. aegypti* genome, the closest *Ae. albopictus* relative that has been sequenced. The choice was appropriate since there is 60-80% average similarity at nucleotide level in genes between these two related mosquitoes, allowing identification of transcripts as well as the identification of vector interspecies differences.

There were certainly restrictions in this study, for example, no other time points were analysed. However the study gives us a general idea of what happens in the cell at a transcription level during viral infection. Our aim was to identify differentially expressed genes so they could be silenced, in the long term, their effect on SFV infection verified, and to generate some sequence information for *Ae. albopictus* generally.

Contig coverage was chosen over transcript number due to the sequencing limiations. 454 sequencing can start indiscriminately from the 3' or from the 5' end of the transcript. If the transcript is degraded or cut at some point, then the read will appear as two different copies of at the same gene. Long RNA transcripts are quite fragile and during the process of preparing the sample for sequencing some will break. It can be assumed that contig coverage is a good indicator of the amount of transcript since, when normalizing, the amount of 40S ribosomal protein 7S transcripts was only 25.65% higher in the infected cells. This means there was only a quarter of a fold difference in transcript level that had to be corrected.

It is known that SFV infection of U4.4 follows a timeline with an acute phase lasting up to 16-24 h p.i. followed by a persistent infection marked by a lower virus production (Fragkoudis et al., 2008b). Since funds for this transcriptomic studies were limited, only one time point was analysed. The time point chosen was at 16 h post-infection, towards the end of the acute phase. Genes involved in controlling SFV infection would have been induced at this time or otherwise responding to infection. It can be assumed that the profile from differentially expressed gene transcripts would vary depending of the stage of infection. Most transcriptomic studies verify sequencing data with some other methods, such as PCR, qPCR or northern blot. Verification of the data was not carried out because of limited time. Contig coverage gives a very high quality quantified amount of RNA bases per transcripts so PCR

verification was not a priority. The next step would have been silencing of up-regulated genes with RNAi. Several candidate genes were going to be silenced and their effect on SFV infection monitored. This was not done since it was decided to follow the role of the melanisation pathway in SFV infection (see following chapters). Potential elements of this pathway, such as serpins, together with genes involved in oxidative stress such as peroxidases and oxireductases, were shown to be highly differentially expressed upon SFV infection in this study.

There is little knowledge regarding the amount or percentage of viral RNA in infected mosquito cells in relation to cellular/host RNA. In mammalian systems, some studies suggest that the percentage of viral RNA can vary over infection duration but it is between 20-40% of the total cellular RNA in influenza infected eggs (Varich et al., 1981). In insects including mosquitoes, the percentage of viral transcripts as of total cellular RNA is unknown. The low percentage of virus transcripts found also relates to discoveries made in the small RNA field. Several recent studies suggest that virus induced siRNAs in mosquito cells, also known as viRNAs, make up a relatively low percentage of the total infected cell sRNAs pool: in WNV (0.43%), SINV (4.79%) LACV(8.39%), and in SFV (2%) (Brackney et al., 2010; Siu et al., 2011b).

There was a general downregulation of gene expression during SFV infection. This correlates with findings from other transcriptome studies with DENV and ONNV in *Ae. aegypti* and *A. gambiae* (Bartholomay et al., 2004; Colpitts et al., 2011; Sim and Dimopoulos, 2010; Sim, Ramirez, and Dimopoulos, 2012; Waldock, Olson, and Christophides, 2012). Experimentally SFV has proven to induce a mild shut down in *Ae. albopictus* derived cells (Fragkoudis et al., 2008c). Gene expression is also tissue specific, and though the U4.4 cell culture system is quite heterogeneous, it lacks the specialisation of individual tissues. In recent times there has been an increase of transcriptome analysis of specific tissues, especially those important in arbovirus infection of mosquitoes, such as the salivary glands or the midgut. It has been found that there is a tissue specific differential gene expression in *Ae. aegypti* mosquitoes infected with WNV, YFV and DENV (Colpitts et al., 2011; Zou et al., 2011).

Some of the differential gene expression observed here correlates with findings from a previous study involving a much less deep transcriptome analysis of *Ae. albopictus* derived C6/36 cells stimulated with bacteria (Dixit, Patole, and Shouche, 2011). However, for our purpose, U4.4 cells are more apt since, unlike C6/36 cells, they have an intact RNAi system

and Imd, Jak/Stat and Toll pathways (Brackney et al., 2010; Fragkoudis et al., 2008b). U4.4 cells therefore represent a more realistic study system.

Though RNAi is the most important anti-viral immune pathway in mosquitoes, RNAi associated genes are largely absent in this study. Dicer-2 transcripts were present but there are not significant differences between samples. The reason this could be is that RNAi components are not differentially regulated upon SFV infection and also present at given time. Other components of classic anti-viral pathways such as Toll and IMD were not differentially regulated in this transcriptome, for example Spätzle 1A (Spz1A) was only up-regulated 0.78 fold in infected cells. This is different in DENV, YFV and WNV infection of Aag2 cells and *Ae. aegypiti* mosquites, where the signalling pathways are heavily up-regulated (Colpitts et al., 2011; Sim and Dimopoulos, 2010).

In this study showed several proteasome and chaperon subunits and regulatory units are differentially expressed in SFV infection of U4.4 cells. There is enough evidence to suggest that the unfolded protein response and ER stress play a role in SFV and DENV infection of mammalian cells (Barry et al., 2010; Doolittle and Gomez, 2011), however this topic is not understood in anti-viral immunity in mosquitoes. Down-reguation of genes that are inhibitors of reactive oxygen species, such as glutathione, is evident in SFV infection of U4.4. Active oxygen species are known to have virucidal properties (Tuladhar et al., 2012) and in insects they are also by-products of melanisation (Kumar et al., 2003). As it will be shown later, melanisation is an antiviral immune response against viruses in mosquitoes.

Taken together, this experiment revealed intriguing patterns of differential transcript abundance that suggest a broad impact of SFV infection in U4.4 cells, such as in metabolism, cell structure and nucleic acid processing. SFV infection induces differential expression of genes in pathways such as apoptosis, stress response and cell cycle. Several studies have tried to illuminate the possible antiviral role of these pathways in mosquitoes upon virus infection, but it is still largely unclear (Wang et al., 2008a; Wang et al., 2012).

As compared to other previously published transcriptome studies, the differential expression of SFV infected U4.4 cells shows a very similar profile to that of ONNV infection of *A. gambiae* cells (Waldock, Olson, and Christophides, 2012) and DENV, YFV and WNV infection of *Ae. aegypti* cells and mosquitoes (Colpitts et al., 2011; Sim and Dimopoulos, 2010; Sim, Ramirez, and Dimopoulos, 2012), where heatshock proteins, serine proteases and

oxidative stress molecules were up regulated and signalling pathways were down regulated. In SFV infection of U4.4 cells however transcripts from signalling pathways genes are not differentially regulated. Similar findings were observed in ONNV infection of *A. gambiae* mosquitoes (Sim et al., 2005).

It is clear that, when analysing the transcriptome data in this study, pathways and gene ontology cannot stand alone. Successful response against viral infection requires a more elaborate gene interactive network. It has been suggested that cross talk between the different immunity pathways, as well as with some non-immune networks, such as stress responses and cell cycle modulation, are essential to mount an immune defence against viruses (Behura et al., 2011). The magnitude and intrinsic nature of these networks is such that a lot more resources and research will be needed to understand the cellular mechanisms behind arbovirus defence in mosquitoes.

Summary of findings

- Most cellular transcripts are not differentially regulated. Only 2% of gene transcripts were up-regulated and 4% of gene transcripts were down-regulated.
- Transcripts up-regulated transcripts upon SFV infection of U4.4 cells were related to nucleic acid processing and serine proteases, as well as other immune related genes, unfolded protein response genes and other stress responses
- Transcripts down-regulated upon SFV infection were involved in exocytosis and vesicle export pathways; RNA degradation, and reactive oxygen species metabolism.

Chapter 4. The role of melanisation as an immune response infection of *Ae. albopictus* derived U4.4 cells

INTRODUCTION

After analysing the results from the transcriptome studies, it was decided to investigate the importance of melanisation and the phenoloxidase ((PO) cascade in SFV infection of U4.4 cells. The phenoloxidase cascade is a very important humoral component of the insect immune system and regulates the formation of melanin following wounding or infection (Cerenius and Söderhäll, 2004; Kanost, 2008). This reaction can be triggered by pathogen-associated patterns (PAMPs) including microbial compounds such as peptidoglycans and LPS, which bind to host microbial pattern recognition receptors (PRRS) and result in the activation the PO cascade. This cascade consists of multiple clip-domain serine proteases and culminates with the activation of prophenolxidase-activating proteases (PAPs) that process the zymogen prophenolxidase (PPO) to active PO. PO then catalyses the conversion of mono- and di-phenolic substrates present in the haemolymph to intermediate products like 5,6-dihydroxyindole or dopamine, which are then further converted into melanin. By-products of PO activity are reactive oxygen species (ROS) that are potent antimicrobial and cytotoxic agents (Cerenius and Söderhäll, 2004). The final products of the PO cascade are largely cytotoxic. This cascade is negatively regulated by host factors including multiple serine proteases inhibitors (serpins) which inhibit the clip-domain serine proteinases in the PO cascade (Cerenius, Lee, and Söderhäll, 2008; Cerenius and Söderhäll, 2004; Christensen et al., 2005; Marmaras and Lampropoulou, 2009; Tang, 2009).

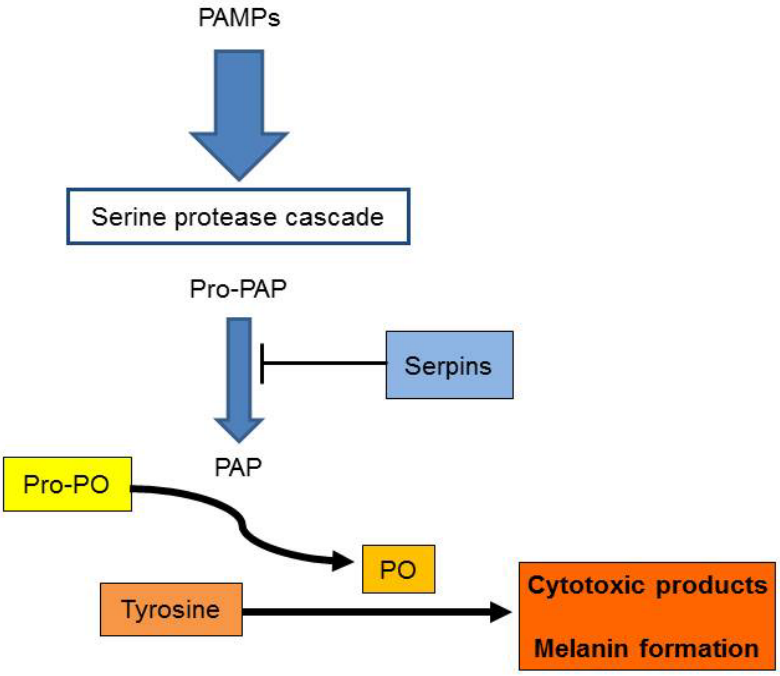

Fig.4.1. The melanisation pathway. PAMPs trigger a serine protease cascade which cleave and activate phenoloxidase activating proteins (PAPs). These molecules then activate PPO into PO which oxidates tyrosine into dopamine prior to further conversion into cytotoxic products, active oxygen species and melanin polymerisation.

PO cascade proteins are found extensively in arthropods and plants (Ashida and Brey, 1995; Cerenius, Lee, and Söderhäll, 2008; Kanost, Jiang, and Yu, 2004; Mayer, 2006). In plants, a PO like protein can induce food browning after activation (Mayer, 2006). In insects, the PO precursor PPO is usually produced by different haemocyte cell types. In Lepidoptera, it is produced by oenocytoids (Ashida and Brey, 1995) and in drosophila it is produced in lamellocytes and crystal cells (Irving et al., 2005; Tang, 2009). Interestingly, in mosquitoes, the production of PPO is more complex and left to specific cell types. Studies in *C. pipiens* suggest that, during larval developmental stages, plasmocytes and the smallest proheamocytes produce PPO. However, after blood feeding in adults, the haemocyte cells

producing PPO are granulocytes and oenocytoids (Wang et al., 2011) (Castillo, Robertson, and Strand, 2006). Such a specialized production of PPO suggests melanisation is important when fighting pathogens in mosquitoes. Moreover, the number of PPO genes varies across insect species. In Lepidoptera, there are two PPO genes (Ashida and Brey, 1995), drosophila has three (Irving et al., 2005) and mosquitoes are known to encode many more; *An. gambiae* encodes nine and *A. aegypti* express up to 10 (Zou et al., 2008b; Zou et al., 2010). It is still unknown why insects like mosquito express a variety PPO genes and if these different genes have diverse properties because of the difficulty to purify and isolate one PPO from another (Chen et al., 2012). However, it is tempting to think that they are involved in controlling a wide range of pathogens in the insect.

In drosophila, melanisation can be activated by pathogens in several distinct tissues, such as the haemolymph, fat body, midgut, trachea or respiratory system as well as in other epithelial tissues (Tang, 2009; Tang et al., 2008). There are limited studies about the PO cascade in mosquitoes, although what is known suggests that this pathway seems to be active and complex (Ashida, Kinoshita, and Brey, 1990). In mosquitoes, pathogen induced melanisation can occur in the haemolymph, midgut and thoracic musculature. Although cellular melanisation is not common, it is mostly a humoral reaction (Hillyer, Schmidt, and Christensen, 2003).

Previous studies implicate the deposition of melanin in defence against fungal infection, multicellular parasites and bacteria (Soderhall and Cerenius, 1998). However, little is known about the role of the PO cascade as an antiviral immune mechanism. It has been suggested that the plasma from the lepidopteran *Heliothis virescens* (tobacco budworm moth) contains factors with antiviral activity against *Helicoperva zea* single capsid nucleopolyhedrovirus (*Hz*SNPV)(most likely linked to phenoloxidase activity) and other viruses including SINV.

Also, bioassays with 5,6-dihydroxyindole show that it rapidly inactivates *Autographa californica* nucleopolyhendrosis virus (AcNPV) (Popham et al., 2004) (Ourth and Renis, 1993; Shelby and Popham, 2006; Zhao et al., 2011). Reactive intermediates, such as 5,6-dihydroxyindole, have been shown to be cytotoxic (Zhao et al., 2011). Antiviral activity against the polydnavirus MdBV has also been correlated with hemolymph melanisation in Lepidoptera (Beck and Strand, 2007). In the case of arboviruses, it is interesting to note that knockdown of PPO I in the mosquito *Armigeres subalbatus* by a recombinant SINV expressing a dsRNA targeting PPO I resulted in reduced PO activity and higher SINV titres (Tamang et al., 2004). However, it still remains unknown whether arboviruses can activate the PPO cascade in mosquitoes and whether products of the PO cascade exhibit biologically relevant antiviral activity.

Objectives

- To determine whether there is a PO cascade in conditioned cell culture medium from *Ae. albopictus* derived U4.4 cells.
- If there is PO activity, investigate whether SFV can activate the PO cascade in *Ae. albopictus* derived U4.4 cells.
- Investigate if PO activity affects SFV infection of U4.4 cells.

RESULTS

The presence of melanisation in U4.4 cell culture medium

Mosquitoes have a functional PO cascade which is an important part of their innate immunity. The haemolymph of *Ae. aegypti* readily melanises in response to wounding and infection. They also encode multiple PPO genes, some of which some are expressed in response to bacterial infection (Zou et al., 2008a). Multiple PPO genes are also detected in haemocyte-like cell lines derived from *Anopheles gambiae* (Muller et al., 1999). In contrast, little is known about PO activity in other mosquito cell lines. *Ae. albopictus* U4.4 cells have proven to be a useful model to study innate immune responses against arboviral infection in mosquitoes (Attarzadeh-Yazdi et al., 2009; Fragkoudis et al., 2008a; Siu et al., 2011a) In order to assess if there is any PO activity in the conditioned cell culture medium of U4.4 cells, a PO activity assay was developed based on enzymatic studies (Hall et al., 1995). Cell free culture medium, which had been in contact with the cells for 48 h, was incubated for 10 min. with *E. coli* or purified SFV. *E. coli* was used as a positive control. Conditioned cell culture medium alone was used as a negative control and then assessed for PO activity. A PO activity assay was then carried out (see Materials & Methods for details) which involved the determination of PO activity through absorbance increase in the culture medium due to dopamine oxidation (Fig.4.1).

These assays revealed significantly higher levels of PO activity in conditioned medium challenged with *E. coli* than in conditioned medium alone (General Linear Model, P=0.004) (Fig. 4.1). Results also showed that purified SFV significantly increased PO activity in conditioned cell culture medium when compared to control conditioned medium (P=0.021) (Fig. 4.1). There was no significant difference between *E. coli* and SFV induced PO activation. It can therefore be stated that SFV virions, like *E. coli*, are activators of the PO cascade.

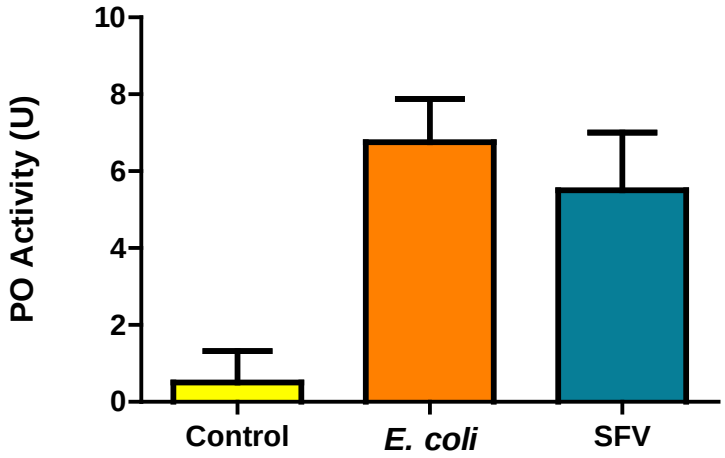

Fig.4.1. PO activity in U4.4 conditioned cell culture medium. PO activity was measured through absorbance due to the oxidation of dopamine in conditioned medium when *E. coli* or purified SFV were added. One unit of PO activity was defined as ΔA490=0.001 after 30 minutes incubation; absorbance changes due to the oxidation of the dopamine substrate by PO activity (see Materials and Methods). The Y axis indicates PO activity units (U). Each bar represents the mean from 10 reactions; error bars represent standard deviation. The experiment was repeated three times with similar results.

SFV virion viability in U4.4 cell culture medium

In the polydnavirus MdBV infection of *M. sexta*, melanisation reduces virus viability by three logs and inhibition of this pathway prevents this effect (Beck and Strand, 2007). In order to assess if potential PO activity in U4.4 cell culture affects SFV viability, a simple preliminary experiment was designed. A total of 2 x 10^8 PFU ml of SFV (titrated in BHK-21 cells) was incubated for 1 h at 28 °C with conditioned or unconditioned U4.4 cell culture medium. Afterwards, both the conditioned and unconditioned culture media containing the virus were titrated.

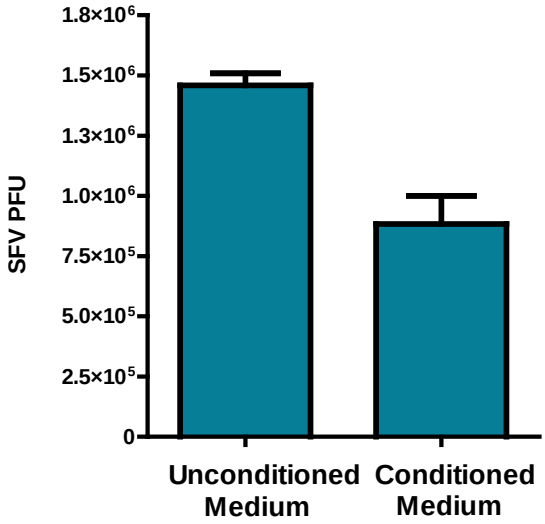

Fig.4.3. SFV viability after incubation with conditioned or unconditioned U4.4 cell-free culture medium. Viability was then determined by titration of SFV on BHK-21 cells. PFU: plaque forming units. Each bar represents the mean from triplicate incubations; error bars show standard deviation. This experiment was repeated three times with similar results.

Virion viability is reduced by almost 50% in conditioned medium. This correlates with findings with MdBV virus (Beck and Strand, 2007). Together with the other findings in this chapter this suggests that PO activity can reduce virion viability.

Identifiying the source of PO activity in U4.4 cell culture medium

Ae. albopictus derived U4.4 is a heterogeneous cell line, it contains cells of different morphologies and origins, some of them possibly haemocytes. It is known that PPO genes are expressed in haemocytes in mosquitoes, in particular granulocytes and oenocytoids (Castillo, Robertson, and Strand, 2006). This experiment was done to determine whether cells in the U4.4 culture express and secret PPO and thus contribute to the PO cascade in conditioned culture medium. U4.4 cells were seeded and after 24 h the cell culture medium was removed and the monolayer was washed with glacial methanol. Methanol and other amphipathic molecules activate the PO cascade (Kanost, 2008) The monolayer was then covered with the PO activity assay substrate buffer (50 mM Sodium Phosphate buffer (pH 6.5) containing 2 mM dopamine) and the cells were incubated for 1 h at room temperature in the dark. Cells expressing PPO genes will stain dark due to the PO activity on the dopamine containing buffer.

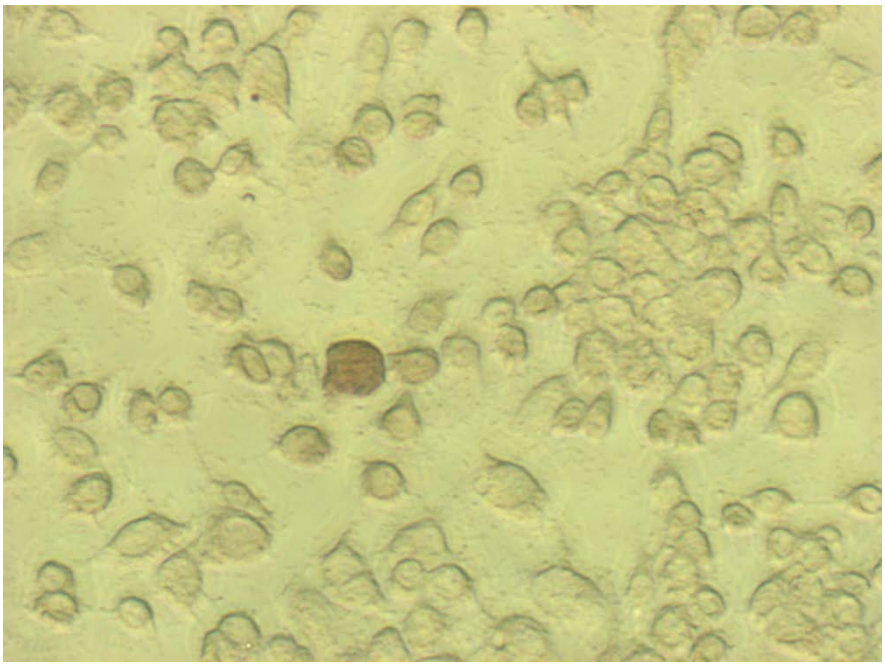

Fig.4.4. PPO producing cells in U4.4 cell culture. U4.4 cells were fixed with glacial methanol. PO activity assay buffer was then added, the culture was incubated for 1 h at room temperature and placed under the light microscope for observation of PO positive cells. This experiment is representative of three independent experiments.

As seen in Fig.4.4, most of the cells were not melanised. A small proportion of melanised cells, as little as approximately 0.2% are probably haemocytes which carry PPOs in vesicles ready to secret them. The type of haemocytes which constitutively express PPO genes are known as oenocytoids. They are morphologically round and bigger as well as they stain positive for PO activity (Castillo, Robertson, and Strand, 2006). This result revalidates that melanisation occurs in U4.4 cell culture, as well as indicates that a small population of specialised cells that also show the features of oenocytoids are responsible for the expression of PPO. Indeed, PPO-expressing haemocytes are just a small proportion of the plasma cells (1% or less) (Castillo, Robertson, and Strand, 2006).

The effect of glutathione (GSH) in counteracting melanisation in U4.4 cell culture medium.

Glutathione (GSH) is a tripeptide thiol found in all the metazoans which exists in both reduced (GSH) and oxidized (GSSG) forms. In mammals it protects cells from the destructive effects of reactive oxygen species and free radicals (Meister and Anderson, 1983). In insects, GSH has been linked to oxidative stress and aging (Jovanović-Galović et al., 2004; Lipke and Chalkley, 1962; Sohal, Arnold, and Orr, 1990). It is produced, exported to the haemolymph and regulated by the haemocytes. These cells are also capable of recycling oxidized glutathione (GSSG) to active GSH. Melanisation does not occur in the insect plasma until secreted GSH levels fall below 20 µM. GSH is a potent inhibitor of PO activity and melanisation. (Clark, Lu, and Strand, 2010).

GSH is used to inhibit melanisation by adding it to the U4.4 cell culture medium. GSH is a naturally occurring peptide in cells, so it was assumed that it had low toxicity. Nevertheless an experiment was carried out to assess the highest concentration of GSH that could be added to the cells without causing a loss in cell viability. U4.4 cells were transfected with a plasmid expressing *Renilla* luciferase under a baculovirus promoter (pAcIE1-R*Luc*) (Ongus et al., 2006) and incubated for 48 h, with cell culture medium containing different concentrations of GSH.

GSH does not seem to affect cell viability until reaching very high concentrations such as 1 or 2mM (Fig 4.5). As mentioned before 20 µM is enough to inhibit melanisation but, since our experiments can take 48 h, a concentration of 0.5 mM was used in experiments to make sure melanisation was inhibited.

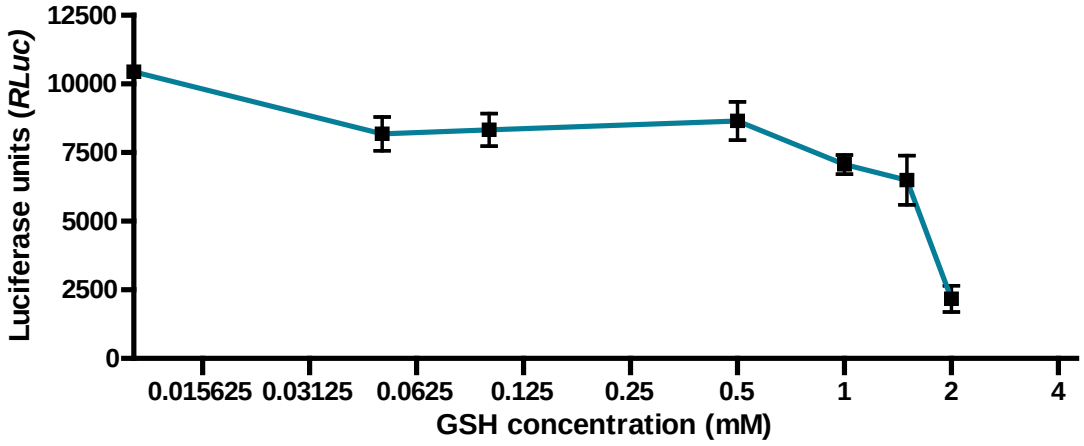

Fig. 4.5. U4.4 cell viability in different concentrations of GSH. U4.4 cells were transfected with pAcIE1-R*luc* plasmid and cultured with different concentrations of GSH added to the medium. *RLuc* readings were taken after 48 h. Each bar represents the mean from triplicate cultures; error bars represent standard deviation. This experiment is representative of three independent experiments.

In the following experiment, the effect of GSH as PO activity inhibitor on SFV infection of U4.4 cells was analysed. U4.4 cells were infected with SFV expressing *Renilla* luciferase (*RLuc*) (SFV4(3H)-*RLuc*) at low MOI (0.005), thus allowing the virus to spread through the culture. GSH was added to the cell culture medium to a final concentration of 0.5 mM and the luciferase readings, indicators of viral replication and spread, were taken after 48 h.

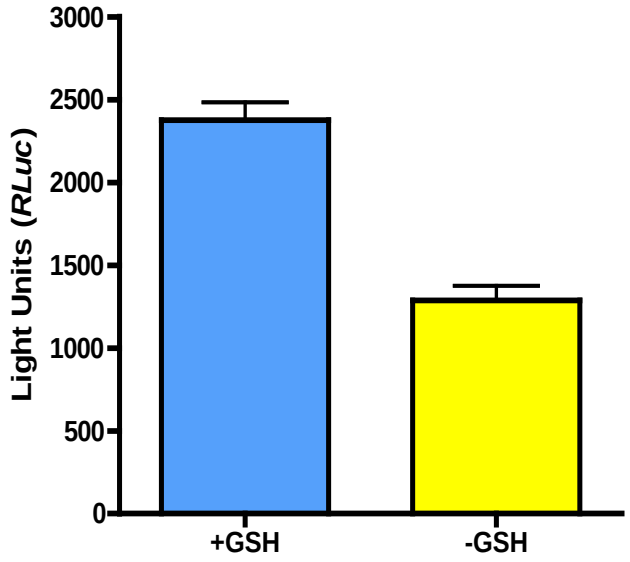

Fig 4.6. Effect of the PO activity inhibitor gluthatione (GSH) on SFV infection of U4.4 cell culture. Cells were infected with SFV4(3H)-*RLuc* at low MOI (0.005)(allowing virus spread to uninfected cells through cell culture medium and multiple rounds of replication) and treated or not with 0.5 mM GSH. *RLuc* readings were taken 48 h post-infection; Y axis indicates light units. Each bar represents the mean from triplicate cultures; error bars represent standard deviation. This experiment is representative of three independent experiments.

The addition of GSH has a positive effect on viral spread. SFV replicates and spreads through the culture significantly better (t-test, P value= 0.0014) than virus in culture medium without GSH. Since GSH inhibits melanisation and does not appear to have another negative effect in cell metabolism, it can be assumed that inhibition of melanisation by GSH favours viral spread through the culture.

DISCUSSION

Melanisation is an important immune defence against bacterial, fungal and parasitic infection in mosquitos and other arthropods. Here I demonstrate that the antimicrobial properties of this pathway also extend to arboviral infection of mosquito cell cultures. I also show that cell conditioned culture medium from *Ae. albopictus* derived U4.4 cells contains an active PO cascade which can be triggered by SFV virions or *E. coli*. PO activity affects SFV virion viability and affects virus spread in cell culture. As it has been previously speculated for *An. gambiae* cell lines (Muller et al., 1999), some cells in the U4.4 cell population seem to produce PPO and have haemocyte-like properties. The presence of glutathione counteracts the effects of the PO cascade during SFV infection of U4.4 cells.

The mechanisms by which SFV triggers melanisation are still unknown. The extracellular soluble particle recognition proteins that activate the cascade when in contact with PAMPs remain largely uncharacterised and but there are several candidate proteins involved. It is believed that immulectins 1 and 2 play an important role in triggering melanisation in the tobacco hornworm *Manduca sexta* (Ling and Yu, 2006; Yu and Kanost, 2004). Immulectins are members of the C-type (calcium-dependent) lectin superfamily. They are produced in haemocytes and function as humoral pattern recognition receptors binding to parasites, such as *C. elegans* and to the human filarial nematode *Brugia malayi*, inducing melanisation and encapsulation (Ling and Yu, 2006). Gram-negative bacteria can trigger melanisation through another type of extracellular receptor, peptidoglycan-recognition protein (PGRP)-LE, which consists of 12 members in *Drosophila*, some of them expressed extracellulary (Werner et al., 2000). Interestingly, in addition to the activation of the PO cascade in the haemolymph, PGRP-LE can also activate an IMD-mediated antibacterial response in *Drosophila* (Takehana et al., 2002). Other molecules that have been linked to activation of melanisation are β-1,3-glucan recognition proteins (GRP) which exist on the surface of bacteria, independently of their Gram-trype, and fungi. Pathogen cell walls can be detected by GRP which are constitutively expressed in *Ar. subalbatus* and, once activated, trigger the proteolytic cascade leading to melanisation (Wang et al., 2005a; Wang et al., 2006). Although there is speculation as to which receptors are involved in the induction of melanisation by parasites and bacteria, the receptors recognising viruses are not known at this stage. The affinity of the previously mentioned receptors to the glycoproteins in the virion has not been tested. It is tempting to hypothesise that this unknown virus receptor will not be specific and could be

activated by several virus families, and that glycoproteins (clustered on the virion) are recognised by a humoral receptor.

How the PO cascade negatively affects the virus infection and spread it is also not known. One possibility is the melanisation and encapsulation of virions though this is difficult to observe. A possible mechanism is the destruction of the virion are reactive oxygen species (ROS) intermediates of the PO cascade, such as 5,6-dihydroxyndole, which have shown to mediate antiviral activity (Popham et al., 2004). The lethal role of ROS against parasites, bacteria and fungi as well as the underlining biochemical mechanisms have been reviewed extensively (Nappi, Poirié, and Carton, 2009). Elevated levels of ROS cause tissue damage by various mechanisms, such as nucleic acid damage, lipid peroxidation, sulfhydryl oxidation and protein cross-linking. Species like oxygen peroxide (*OH) are able to react with any organic molecules and its production requires the reduction of certain transition metals, like Cu^{2+} and Fe^{3+}, as well as interactions with nitrogen peroxide (*NO). Fe^{3+} and *NO unregulation has been linked to mediating various toxic and antimicriobial responses in arthropods (Foley and O'Farrell, 2003; Kremer et al., 2009). In the host, a good balance and tight regulation of the production of ROS is needed in order to avoid the generation of cytotoxic compounds at nonspecific sites within the host hemocoel (Nappi, Poirié, and Carton, 2009). Molecules like the previously mentioned glutathione GSH are responsible for controlling ROS. Addition of GSH to the cell culture, thus removing ROS, resulted in an increase of viral infection and spread. Therefore, it can be hypothesised that melanisation affects the virus by ROS and cytotoxic agent production, however, the specific biochemical mechanisms of the interaction between these molecules and the virion are not known.

Melanisation in response to virus infection of mosquitoes may be important as an immune response, however, other inducible pathways such as Toll, IMD and, more importantly RNAi are also key to controlling the arboviruses. This will be explored further in future chapters. One interesting and significant aspect of melanisation is not just that the pathway itself affects the virus, but that, independent of how efficient the PO cascade is, it can also trigger other immune pathways. In drosophila, tracheal melanisation induces systemic expression of the antimicrobial peptide drosomycin, an end product of the Toll and IMD pathways, in the fly's fat body (Tang et al., 2008). Induction of melanisation and cross talk with the Toll pathway has also been suggested in *Ae. aegypti* infection with Wolbachia and DENV (Rancès et al., 2012). However, the mechanisms linking melanisation with the Toll pathway and the

production of antimicrobial peptides are still obscure (Ligoxygakis et al., 2002; Scherfer et al., 2006). As mentioned before, PRG-LE activated receptors can also trigger anti-bacterial IMD/relish induced transcripts (Takehana et al., 2002). However, with the exception of drosomycin, nothing is known about the induction of anti-viral peptides and other antiviral activities. ROS produced during melanisation can also activate NF-κB due to pro-inflammatory cytokine release (Nappi, Poirié, and Carton, 2009). Activation of NF-κB seems to be important for virus infection in arthropods since several polydnaviruses have evolved inhibitors against these molecules (Thoetkiattikul, Beck, and Strand, 2005).

This chapter demonstrates the importance of the PO cascade as an innate response against viral infection of mosquito cells and this will be further explored, however there are still several unknown aspects of this pathway. To add complexity, in mosquitoes several distinct melanisation pathways exist and the *in vivo* mechanisms of this cascade following virus infection may be even more intricate (Zou et al., 2010). In the following chapters the role of melanisation during virus infection will be studied further using melanisation inhibitors. Also, the role that serine protease inhibitors (serpins) have in the PO cascade during viral infection will be investigated.

Summary of findings

- There is a melanisation cascade and PO activity in U4.4 cell conditioned medium.

- SFV and *E. coli* can activate the PO cascade in U4.4 cell culture medium.

- SFV replication and spread is affected by intermediates of the PO cascade.

Chapter 5. SFV expression of the melanisation inhibitor Egf1.0 and its effects on the PO cascade

Introduction

Melanisation is a highly conserved pathway across insect families, key enzymes of this cascade do not differ excessively across insect families. This has allowed parasitoid wasps, thanks to the expression of PDV proteins (Lu *et al.*, 2008), to overcome the immunity of this pathway while depositing their eggs across a great variety of insect species larvae as described in Chapter 1. *Microplitis demolitor* bracovirus (MdBV) is carried by the braconid wasp *M. demolitor* and allows the wasp to parasitize the larval stage of several Lepidoptera species (Kadash *et al.*, 2003). The genome of MdBV is 189 kb long, divided in 15 segments and encodes for 61 predicted genes (Webb *et al.*, 2006). Most of these genes belong to four families, mucin-like cell surface genes (Glc family), IκB-like genes (Vankyrins), protein tyrosine phosphatase (PTP) genes, and epidermal growth factor-like motif genes (Egf family). Previous studies suggest that members of the Glc, Vankyrins and PTP families can disrupt phagocytosis, encapsulation and NF-κB signalling, however they do not affect the host's melanisation response. On the other hand, proteins of the Egf family have shown anti-melanisation properties. The Egf family is composed of 3 proteins, Egf1.5, Egf1.0 and Egf0.4 (Beck & Strand, 2007). While Egf1.5 and Egf1.0 inhibit melanisation the smallest protein, Egf0.4, does not (Lu *et al.*, 2008 and 2010). For this study we have focused on the properties of the Egf1.0 gene product, which has been mainly developed by the Strand group (University of Georgia) using *Manduca sexta* (tobacco hornworm) as a model insect melanisation system.

Egf1.0 is 26.0 kDa protein containing an N-terminal 9 cysteine-rich, trypsin inhibitor-like domain (CD), a secretion signal, and a C-terminus repeat domain (RD) comprised of three nearly identical 35 amino acid repeats arranged in tandem array (Strand *et al.*, 1997). Egf1.0 displays dual-activity inhibition of the PO cascade which suppresses melanisation in two fashions. The first one is by completely inhibiting PAP enzymatic activity via cleavage at its reactive amidolytic site (LCYR*FQQF) by the CD. Secondly, Egf1.0 also blocks activation and processing of pro-PAP1 and pro-PAP3 by competitively binding through the RD in a dose dependent manner (Lu *et al.*, 2008). Taken together these processes suppress the ability of host plasma to melanize. Experiments further indicate that inhibition of melanisation enhances survival of *M. demolitor* and MdBV itself in lepidoptera's haemolymph (Beck & Strand, 2007).

The expression and inhibitory efficiency of Egf1.0 has been tested in *Ae. aegypti* mosquitoes haemolymph to see its effect on parasite inducible melaninsation (Beck and Strand, 2007). In order to investigate the effects of this inhibitor on virus spread and replication in the mosquito system and the effect of PO activating in virus spread, we produced SFV constructs expressing this gene.

The 26S subgenomic promoter which drives the expression of the viral structural proteins is very potent in alphaviruses. To take advantage of the strong, prolonged expression properties of the subgenomic promoter without sacrificing the ability of the virus to replicate productively, viral constructs expressing the foreign gene of interest under the control of a duplicated subgenomic promoter have been constructed and utilized in various studies. Such replication-competent vectors have been described for several alphaviruses including SFV and SINV (Ehrengruber and Lundstrom, 2002).

For more convenient virus production in BHK-21 cells, the SFV vector chosen was based on a CMV promoter backbone (pCVM-SFV4). This construct has a full length infectious cDNA clone of SFV4 under the control of a CMV promoter (pCMV-SFV4) which allows direct transfection of the cDNA into BHK-21 cells instead of RNA, which is more efficient (Ülper et al., 2008).

In the present chapter, several SFV vectors expressing Egf1.0 under a second 26S subgenomic promoter were constructed and characterized *in vivo* and *in vitro*. The effects this inhibitor has on viral replication and spread were investigated.

Objectives

- To clone the Egf1.0 gene into SFV4, and express this PO activity inhibitor in *Ae. albopictus* derived U4.4 cells.
- To functionally test the effect of Egf1.0 expressed by these viral constructs in the melanisation pathway.
- To examine the effect of Egf1.0 on SFV replication and spread in U4.4 cell culture.
- To investigate the effect of Egf1.0 on SFV infection of *Ae. aegypti* mosquitoes.

Construction of recombinant SFV expressing Egf1.0 under a second subgenomic promoter

Recombinant viruses used for this project were constructed at the Tartu University Institute of Technology with the assistance of Margus Varjak and Prof. Andres Merits. A novel SFV vector, annotated as pCMV-SFV4 (Ulper et al, 2008.), was used as the backbone. The use of a CMV promoter backbone has several advantages including simplifying the cloning process, low toxicity in *E. coli* and ease of transfection. A duplicated subgenomic promoter was placed behind the structural proteins as described in Rausalu et al, 2009 The duplicated subgenomic promoter used was T37/17, which is 37 nucleotides upstream and 17 nucleotides downstream of the start-site of the subgenomic mRNA. Expression vector pCMV-SFV4-T37/17 had been established previously by Margus Varjak and Andres Merits. In short, this T37/17 second subgenomic promoter sequence was inserted into pCMV-SFV4 so that a BamHI restriction site could be used for placing the insert. The Egf1.0 sequence fragment was PCR amplified with primers EGF1.0_F and EGF1.0_R in a manner that BamHI restriction sites were added to both, 5' and 3' termini. PCR amplified EGF was cloned into BamHI-digested pCMV-SFV4-T37/17. Since the both ends of PCR fragment had BamHI restriction sites Egf1.0 was cloned in both forward and reverse orientations as negative control (Fig.5.2). The ZsGreen and DsRed markers were inserted into the C-terminal region of nsP3 via a *Xho*I site naturally occuring in the SFV genomic sequence (leading to expression of nsP3 containing ZsGreen), while Firefly luciferase (*FFLuc*) was inserted between duplicated nsP2 cleavage sites at the nsP3/4 junction as a cleavable reporter, using strategies previously shown in Tamberg et al, 2007.

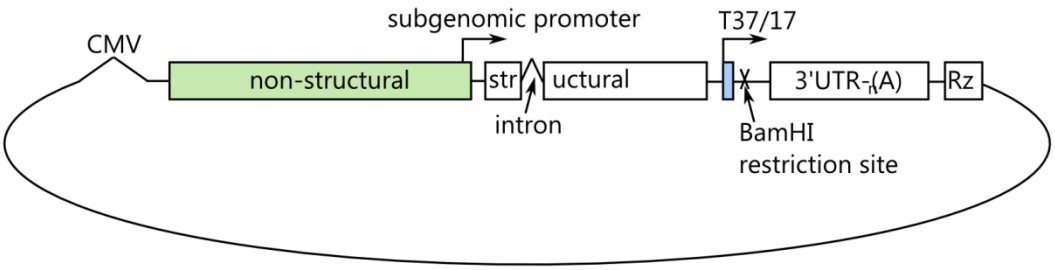

Fig 5.1 Cloning Egf1.0 under a second subgenomic promoter in SFV Semliki Forest virus. The foreign gene is inserted using specific restriction enzyme sites and adapted primers with restriction enzyme recognition sequences. The expression of the foreign gene is regulated by a modified second subgenomic promoter (SG 17/37). The presence of a random 21bp region between the promoter and the foreign gene is essential to ensure the expression of the foreign gene under the second subgenomic promoter**.**

Results

The following 4 SFV-Egf1.0 constructs were produced.

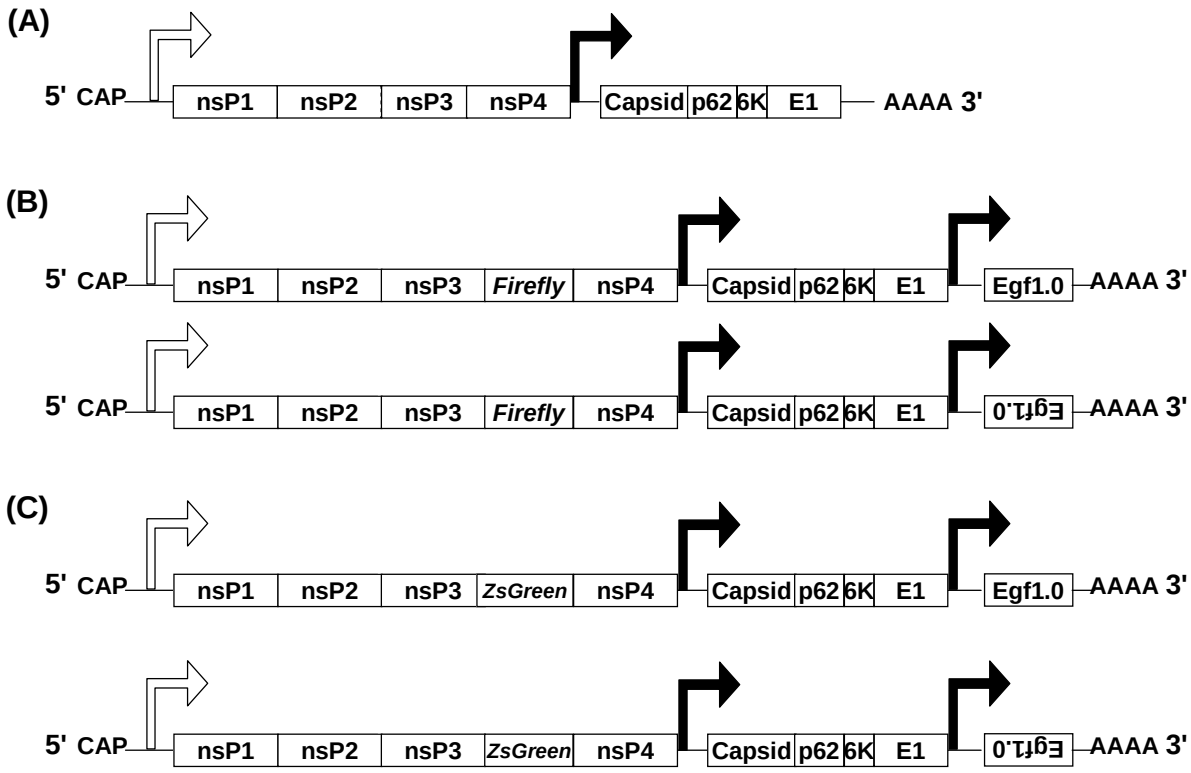

Fig 5.2 Viral constructs used in this study. (A) SFV4, a laboratory prototype strain of SFV. (B) SFV(3H)-FFLuc-Egf1.0F and SFV(3H)-FFLuc-Egf1.0R, encoding Firefly luciferase (FFLuc) as part of the non-structural protein (inserted between duplicated nsP2 cleavage sites at the nsP3/4 junction). The melanisation inhibitor was expressed from a duplicated subgenomic promoter (black arrow) in sense (F virus; top) or in antisense oritentation (R virus; bottom) as a negative control. (C) SFV(3F)-ZsGreen-Egf1.0F or R reporter viruses expressing the fluorescence protein ZsGreen instead of FFLuc. In this case ZsGreen is inserted into the C terminus of nsP3 which enables the visualisation of replicating complexes. A virus expressing DsRed instead of ZsGreen was also created but it was not used due to its inability to produce fluorescence.

Troubleshooting

The *DsRed* virus failed to produce visualisation of replication complexes, even though it was functional. The virus was able to replicate and produce cytopathic effect which was observed in BHK-21 cells when purifying the virus and also when titrated in a plaque assay. *DsRed* works as a tetramer and in this setting it was expected to work as a monomer, like monomeric (m)Cherry. Therefore that seems to be the reason it was not visualised. The rest of the constructs were tested for either *FFLuc* or *ZsGreen* activity. All the clones were sequenced for the correct orientation of Egf1.0. A mutation in this gene was found, however this mutation it was also present in the original plasmid and in the Strand laboratory constructs, who provided us with the clone (Fig.5.3). The functionality of Egf1.0 has been tested with positive result in in vitro and in vivo tests (Beck and Strand, 2007).

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 Identities = 680/681 (99%), Gaps = 0/681 (0%)
 Strand=Plus/Plus

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Fig.5.3 Egf1.0 gene sequence used in cloning (subject) compared with Egf1.0 sequenced found in PubMed database (query). Notice mutation at position 48. This discrepancy is the result of a mistake in the published database entry. Amino acid translation resulted in the entry codon being TT*C* (Phe) instead of TT*G* (Leu). Subject protein functionality has been tested with positive results.

Effect of melanisation on SFV replication and spread dynamics in U4.4 cells

In order to assess the impact that a melanisation inhibitor has on virus replication and spread, *Ae. albopictus* derived U4.4 cell monolayers were infected with SFV4(3H)*FFLuc*-Egf1.0F viruses. The SFV clones with Egf1.0 in a reverse orientation (R viruses) were used as negative controls. Two different multiplicities of infection (MOI) were used; high (MOI 10) and low (MOI 0.005). The high MOI will result in a primary infection of most cells with little chance for virus spread. At the low MOI, virus can spread through the culture thus mimicking *in vivo* infection. Virus spreading through the culture medium from infected to uninfected cells also allows induction of PO activity in conditioned medium. Firefly luciferase expression levels were measured from cell lysates 24 h and 48 h post-infection.

In a similar fashion, this experimental procedure was carried out with viruses carrying ZsGreen fluorescent protein (SFV4(3F)-*ZsGreen*-Egf1.0 F/R) fused to nsP3. This fusion allowed the visualisation of replication complexes after infection. The same MOIs (MOI 10 and 0.005) were used and the cells were fixed at the same two time points (24 h and 48 h) before visualisation by confocal microscopy.

Dectection of viral replication and spread in the presence of absence of Egf1.0 by luciferase assay

At high MOI, the presence of a melanisation inhibitor did not show any effect on viral replication of SFV4(3H)FFLuc-Egf1.0F over SFV4(3H)FFLuc-Egf1.0R at 24 h or 48 h post-infection. Replication rates are higher at 24 h post-infection and drop drastically at 48 h post-infection as the culture enters the persistent phase of infection, as it has been previously described in cell culture (Fragkoudis et al., 2008c) (Fig 5.4). Conversively, at low MOI, the virus expressing the inhibitor showed higher levels of spread and replication at 48 h than the virus without the inhibitor, (GLM, P=0.004) (Fig.5.4). At 24 h post-infection SFV4(3H)FFLuc-Egf1.0F and SFV4(3H)FFLuc-Egf1.0R viruses did not show any significant differences in luciferase levels at any of the two MOIs (GLM, P>0.005).

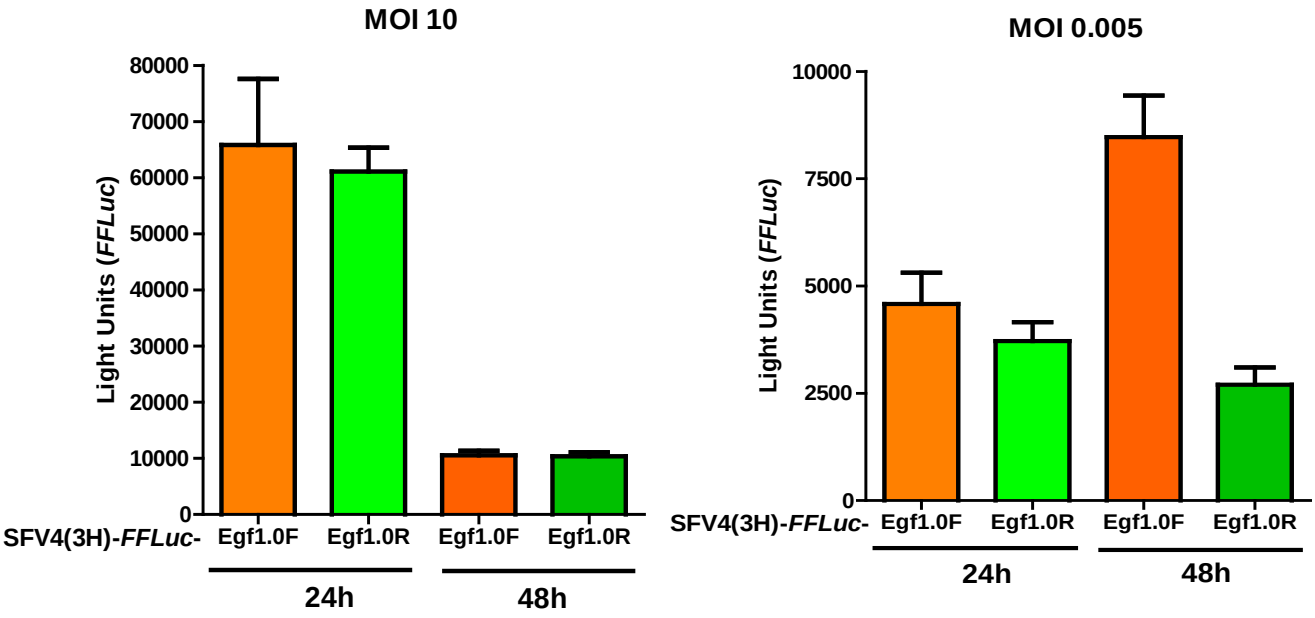

Fig 5.4. SFV expressing *FFLuc* and Egf1.0 either in forward (Egf1.0F) or reverse (as negative control)(Egf1.0R) were used to infected a U4.4 cell culture. Two different MOIs were used, high (MOI 10), mimicking primary infection, and low (MOI 0.005), allowing the virus to spread through the culture. Firefly luciferase was measured as a quantification of viral activity at 24 h and at 48 h. At high MOI the differences between the virus expressing the inhibitor and the one that does not are not significant. However at low MOI at 48 h the virus expressing Egf1.0 produces significantly more luciferase. Each bar represents the mean from triplicate cultures; error bars represent standard deviation. This experiment is representative of three independent experiments.

Dectection of viral replication and spread in the presence or absence of Egf1.0 by confocal microscopy

Viral replication complexes can be visualized as punctuate green cytoplasmic signals due to the nsP3/ZsGreen fusion (Fig.5.5). The expression of green fluorescence corroborates the luciferase findings. At high MOI (10), most of the cells in the culture were infected with either of the viruses SFV4(3F)-*ZsGreen*-Egf1.0F or SFV4(3F)-*ZsGreen*-FFLuc-Egf1.0R, either at 24 h or 48 h. At the low MOI of 0.005, after 48 h, more infected cells were detected with SFV4(3L)-*ZsGreen*-Egf1.0F than with SFV4(3F)-*ZsGreen*-Egf1.0R, thus suggesting that the virus expressing Egf1.0 is able to spread better through U4.4 cell culture.

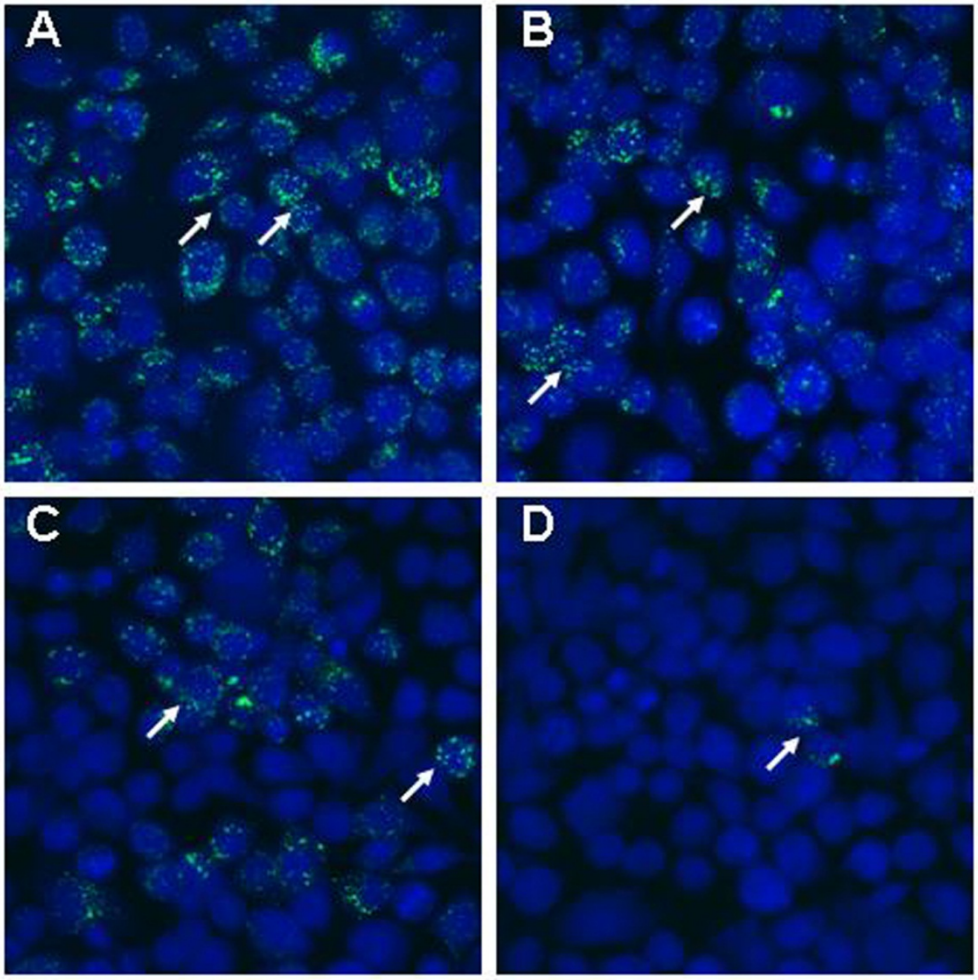

Fig 5.5 Infection of U4.4 cells with Egf1.0 expressing SFV. Cells were infected with SFV4(3F)ZsGreen-Egf1.0F (A, C) or SFV4(3F)ZsGreen-Egf1.0R (B, D) as a negative control. As in the previous experiments, two MOIs were used, high (MOI 10) mimicking primary infection (A, B), and low (MOI 0.005) allowing the virus to spread (C, D). The cells were fixed after 48 h and the cell nuclei were labelled using TOPRO3. The reporter viruses contain ZsGreen as fluorescent marker fused to nsP3; viral replication complexes are visualized as green dots (indicated by arrows). This experiment was repeated three times.

Egf1.0 expression, functionality and characterization in U4.4 cell culture.

A series of experiments were designed in order to further characterise SFV-expressed Egf1.0, in particular expression and secretion. A western blot carried out in order to investigate if recombinant Egf1.0 is expressed from SFV in U4.4 cells. U4.4 cells were infected either with SFV(3F)*FFLuc*-Egf1.0 F, SFV(3F)*FFLuc*-Egf1.0 R at an MOI of 10. After 24 h conditioned cell culture medium was collected. Egf1.0 antiserum was kindly provided by Prof. Michael Strand (University of Georgia, Athens, GA, USA) and used to detect Egf1.0 protein in the cell lysate and conditioned cell culture medium.

Expression of Egf1.0 by SFV4(3H)FFLuc-Egf1.0F but not by control reverse was demonstrated in conditioned cell culture medium and cell lysate, which demonstrated that the recombinant protein was exported from the cell. This confirms our previous results which suggested that Egf1.0 was active in U4.4 cell culture medium (Fig.5.6). Similar experiments were carried out by the Strand lab with extracts of SFV4(3H)-ZsGreen-Egf1.0F or R infected cell lysates or conditioned medium. The same results were obtained (not shown).

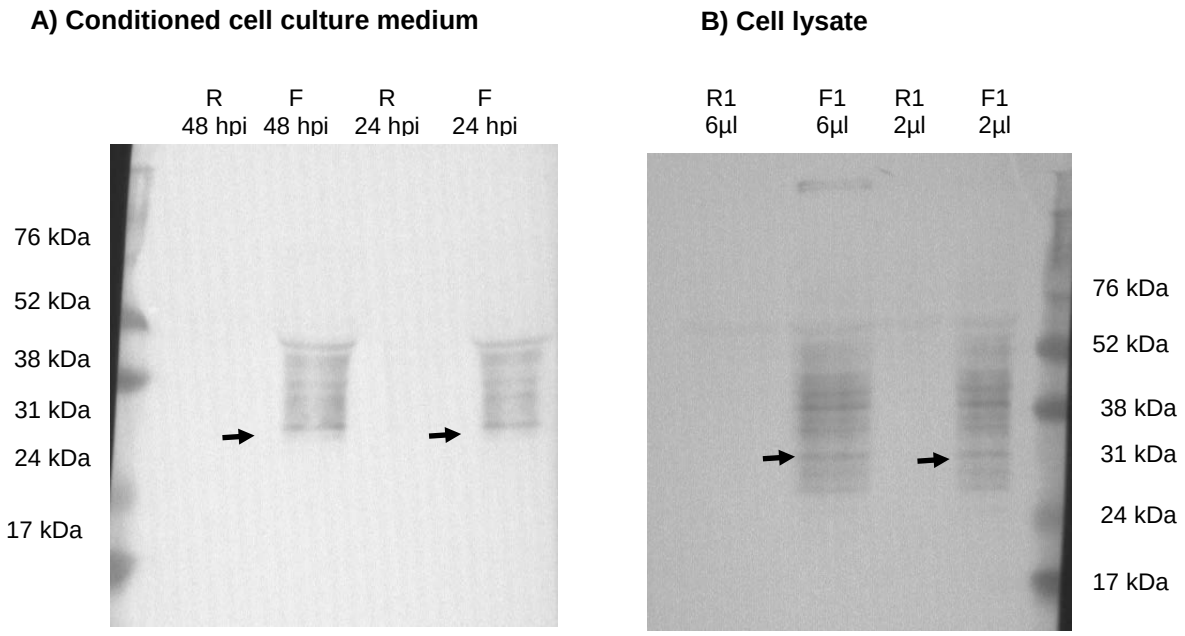

Fig.5.6 Detection of Egf1.0 in conditioned cell culture medium of U4.4 cells infected at high MOI (10) with SFV4(3F)-*FFLuc*-Egf1.0F (F) or SFV4(3F)-*FFLuc*-Egf1.0R (R). Molecular weight markers (Page Ruler Plus pre-stained protein ladder, Thermo Scientific) are indicated. NBT/BCIP detection system was used to detect Egf1.0 in the conditioned cell culture medium (A) or in the cell lysate (B). Egf.10 is ~27kb (indicated by arrow), higher bands indicate different glycosylation stages of this protein. (6µl) and (2µl) indicate the amount of lysate loaded in the well.

Inhibition of PO activity by SFV4 expressed Egf1.0

Previous studies demonstrate that the protein Egf1.0 functions as a PAP inhibitor that disables activation of the PO cascade and melanisation in multiple insect species, including *Ae. aegypti* (Beck and Strand, 2007). The amino acid alignment showed that the proteolytic cleavage sites used by Egf1.0 were indeed conserved between the different PPOs (Fig.5.6). Egf1.0 acts as a competitive inhibitor of the PO cascade in *Ae. aegypti*.

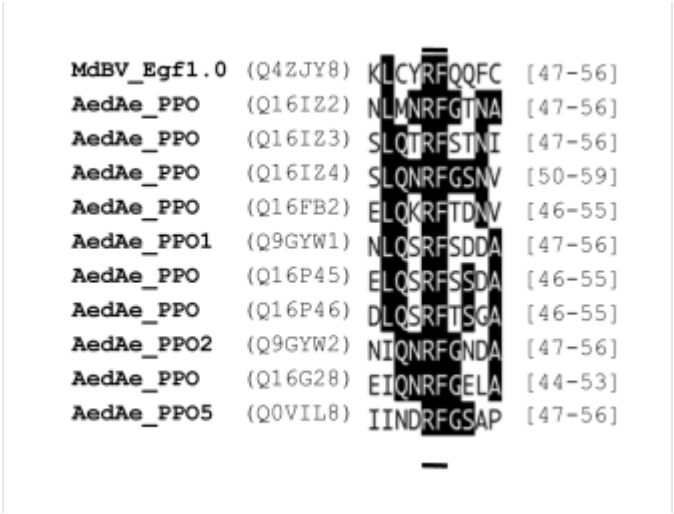

Fig.5.6. Alignment of the reactive site loop of Egf1.0 to the predicted cleavage sites for the PPOs encoded by *Ae. aegypti* (indicated as AedAePPO). Identical P1-P1' residues R-F of Egf1.0 and PPO family members at which cleavage occurs are underlined. Black highlighting indicates identical residues. UniProt database identifiers in parentheses to the left of the alignment. (Provided by Markus Beck, University of Georgia).

Egf1.0 inhibits PO cascade activation in U4.4 cell conditioned medium

Conservation of Egf1.0 PO inhibitory activity was also confirmed in a PO activity assay. U4.4 cells were infected at high MOI (10) either with SFV4(3H)*FFLuc*-Egf1.0F, SFV4(3H)*FFLuc*-Egf1.0R or mock infected. After 48 h post-infection the cell culture medium was collected and the PO activity was measured as described in chapter 4. *E. coli*, was used as a control for activation of PO activity, and the PO activity readings taken.

The ability of recombinant Egf1.0 to inhibit the PO cascade remains intact when expressed from a SFV4 construct. Conditioned U4.4 cell culture medium infected with

SFV4(3H)*FFLuc*-Egf1.0F showed a significant decrease (~75%) in PO activity compared to conditioned medium from cells infected with control virus SFV4(3H)*FFLuc*-Egf1.0R ($P<0.001$) and was not different from PO activity in conditioned medium from the uninfected control cultures ($P=1$). It should also be noted that infection with SFV4(3H)*FFLuc*-Egf1.0R activated the PO cascade when compared to activity recorded from supernatant from uninfected control cells ($P=0.0031$), which corroborates previous findings that virus alone in cell culture medium is sufficient to trigger PO activity. Activators of the melanisation cascade, such as *E. coli*, do not rescue the PO activity in the presence of Egf1.0. Compared to conditioned medium alone from uninfected control cultures, incubation with *E. coli* did not result in significant increase of PO activity in SFV4(3H)*FFLuc*-Egf1.0F infected cell culture medium ($P=0.251$). Incubation of cell culture medium from uninfected control cells with *E. coli* resulted in high PO activity.

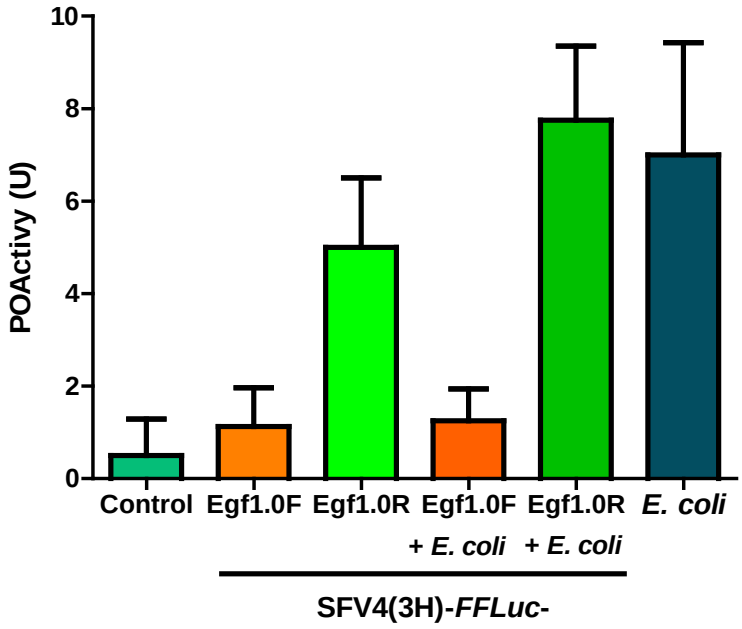

Fig 5.7 PO activity in conditioned cell-free culture medium of U4.4 cells infected with SFV4 expressing or not Egf1.0. 6.5 x 10^5 U4.4 cells were infected with SFV4(3H)-*FFLuc*-Egf1.0F (labelled Egf1.0F), or SFV4(3H)-FFLuc-Egf1.0R (labelled Egf1.0R), at an MOI of 10 or uninfected (labelled control). Medium was collected 48 h p.i. and the PO cascade induced with *E. coli* where indicated; PO activity in samples was determined by measuring increase in absorbance of a dopamine based substrate (see Materials and Methods). Y axis indicates PO activity units (U). Each bar represents the mean from 10 reactions; error bars represent standard deviation. This experiment is representative of three independent experiments.

Effect of Egf1.0 on SFV infection of BHK-21 cells

As a final control experiment, Egf1.0 expression was tested under conditions where melanisation should not have an antiviral role. Melanisation does not take place in vertebrate cells, therefore the constructs expressing Egf1.0 should not have any advantage over those which do not express it.BHK-21 cells were infected with either SFV4(3H)-*FFLuc*-Egf1.0F or SFV4(3H)-*FFLuc*-Egf1.0R at low MOI (0.005), thus allowing for spread of the infection. After 24 and 48 h the cells were lysed and luciferase readings were taken as an indicator of viral replication and spread.No significant differences were observed between SFV4(3H)-*FFLuc*-Egf1.0F and SFV4(3H)-*FFLuc*-Egf1.0R (GLM; P=0.642). Vertebrate cells do not have an equivalent of the melanisation cascade, the Egf1.0 expressing virus did not obtain an advantage in replication in contrast in what we observed in U4.4 cells.

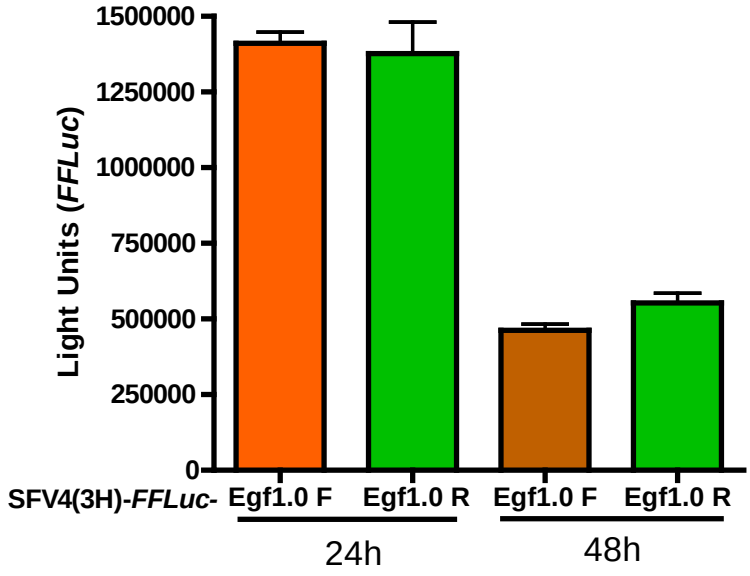

Fig 5.8. Infection of BHK-21 cells with SFV4(3H)-*FFLuc*-Egf1.0F or SFV4(3H)-*FFLuc*-Egf1.0R. Infections were done at a low MOI (MOI 0.005), allowing the virus to spread along the culture. *FFluc* activity was determined at 24 h and 48 h post-infection. Each bar represents the mean from triplicate; error bars represent standard deviation. This experiment is representative of three independent experiments.

The effect of GSH in U4.4 infection with SFV expressing Egf1.0

As it was mentioned previous chapter, GSH inhibits melanisation by reducing quinones (Clark, Lu, and Strand, 2010a). In order to assess the effects of reactive intermediate by-products of the PO cascade in the presence of SFV expressing Egf1.0, U4.4 cells were infected with these viruses at low MOI (0.005) and 0.5mM of GSH were added to the culture medium. After 48 h p.i. *FFLuc* activity was measured. The results show that inhibition of melanisation by adding GSH into the cell culture medium increases the spread of SFV4(3H)-*FFLuc*-Egf1.0R in comparison to medium without the addition of GSH ($p>0.001$). However, it did not alter the spread of SFV4(3H)-*FFLuc*-Egf1.0F ($p=0.139$) (Fig.5.9).

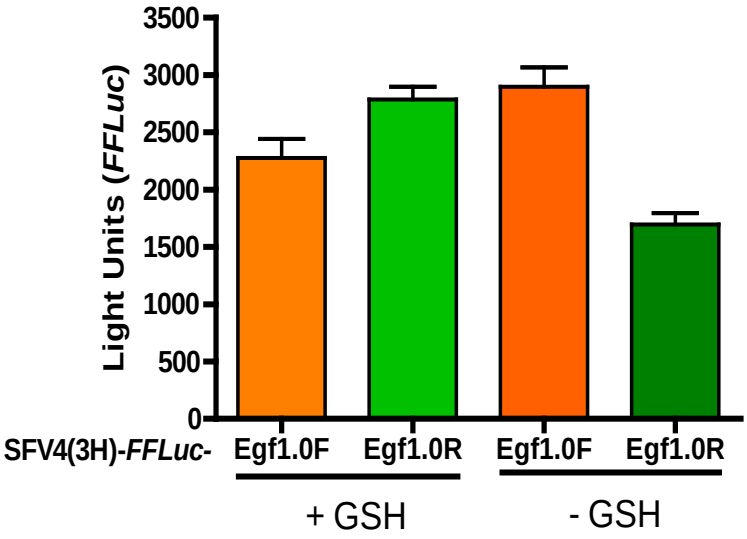

Fig.5.9. Addition to GSH into U4.4 cell culture medium reduces the advantage conferred by Egf1.0 to the virus. 6.5 x 10^5 U4.4 cells were infected at MOI 0.005 either with SFV4(3H)-FFLuc-Egf1.0F or SFV4(3H)-FFLuc-Egf1.0R and GSH (0.5 mM) was added or not to the culture media . Luciferase activity was measured at 48 h. p.i. Each bar represents the mean from triplicate cultures; error bars show standard deviation. This experiment was repeated three times with similar results.

Effect of Egf1.0 expression on SFV4 infection of *Ae. aegypti* mosquitoes

In order to assess the role of the PO cascade in SFV infection of mosquitoes the SFV4-Egf1.0 constructs were tested in *Ae. aegypti* mosquitoes. When inhibiting pathways with antiviral relevance in mosquitoes, by expressing for eample RNAi inhibitors, there has been a reported increase in mosquito mortality. This was proven by infection of a mosquito with an alphavirus expressing RNAi inhibitors (Cirimotich et al., 2009; Myles et al., 2008). *Ae. aegypti* mosquitoes have been shown to be permissive to SFV infection and they are also able to transmit this virus in laboratory conditions (Davies and Yoshpe-Purer, 1954; Nye and Bertram, 1960; Woodall and Bertram, 1959).

Cohorts of *Ae. aegypti* females were fed bloodmeals containing 5 x 10^7 PFU/ml of SFV4(3H)-*FFLuc*-Egf1.0F, SFV4(3H)-*FFLuc*-Egf1.0R, or no virus (mock-infection). Engorged females were separated (cohorts of 22 to 25 mosquitoes) and survival was monitored for the following days indicated (Fig.5.10)

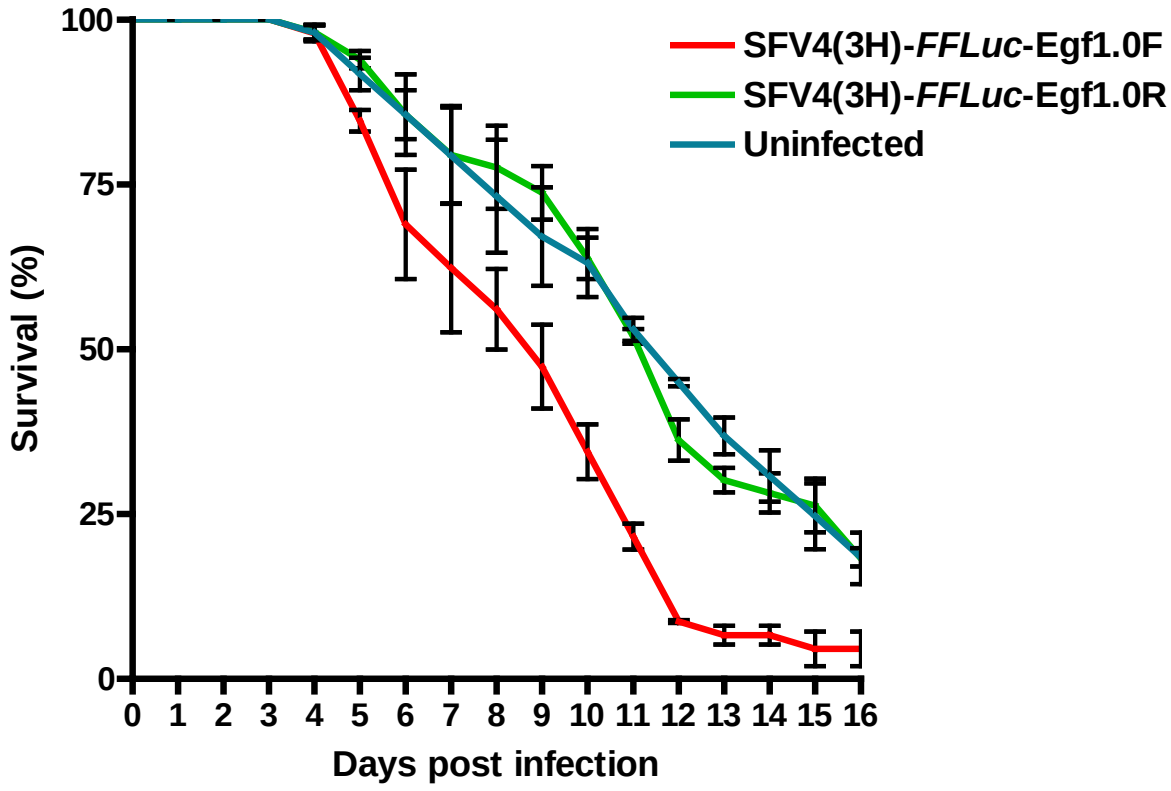

Fig 5.10. Survival of mosquitoes fed with a blood meal containing SFV4(3H)-*FFLuc*-Egf1.0F (red), SFV4(3H)-*FFLuc*-Egf1.0R (green), or no virus (blue). This result shows the combined results of three independent experiments with 22-25 blood-fed female mosquitoes per cohort.

Mosquito survival was significantly dependant on the virus included in the blood meal (Kaplan Meier χ^2=25.37; P<0.001). Mosquitoes fed with blood containing SFV4(3H)-*FFLuc*-Egf1.0F showed a significantly lower survival rate than either the mosquitoes infected with SFV4(3H)-*FFLuc*-Egf1.0R or the mock-infected mosquitoes (P<0.001). Statistical analysis (Post Hoc) showed that that there was no significant difference in survival rates between the mock-infected control and mosquitoes infected with virus which did not express the Egf1.0 inhibitor (P=0.98). Therefore, we can conclude that inhibition of the PO cascade resulted in a negative effect on survival of *Ae. aegypti* mosquitoes.

In order to assess whether the reduced survival of SFV4(3H)-*FFLuc*-Egf1.0F-infected mosquitoes was associated with enhanced viral replication and not to a secondary infection with fungi or bacteria due to the inhibition of the PO cascade a further control experiment was designed.

Cohorts of *Ae. aegypti* female mosquitoes were fed bloodmeals containing SFV4(3H)-*FFLuc*-Egf1.0F or SFV4(3H)-*FFLuc*-Egf1.0R. The blood-fed females were separated and taken for further analysis. Total RNA was then extracted from 10 mosquitoes per cohort at 3 days post-bloodmeal. SFV genome copy number was then determined by qPCR for each individual. The time point at three days post blood-meal was chosen because it just precedes quantifiable differences in mosquito survival and thus avoiding bias induced by mosquito mortality.

Results showed that mosquitoes fed with SFV4(3H)-*FFLuc*-Egf1.0F had higher genome copy numbers than mosquitoes fed with SFV4(3H)-*FFLuc*-Egf1.0R (Mann-Whitney test, p=0.04)(Fig.5.11). Interestingly, infection rates were also higher when mosquitoes were infected with SFV4(3H)-*FFLuc*-Egf1.0F than SFV4(3H)-*FFLuc*-Egf1.0R. This correlates with previous findings where infection rates were higher if alphaviruses expressed RNAi inhibitors (Cirimotich et al., 2009), and suggests that inhibitory PO activity can increase infection rates.

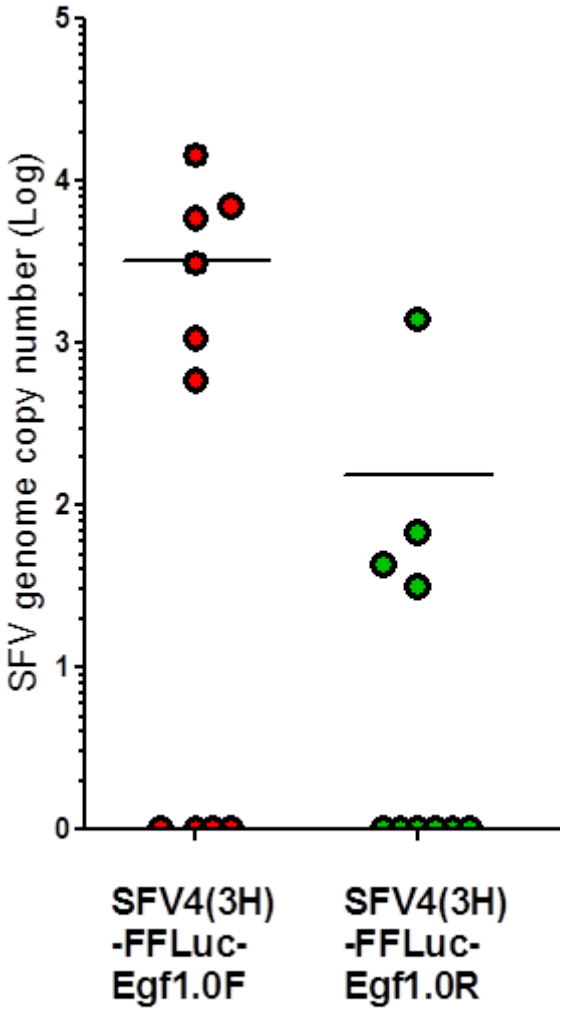

Fig.5.11 SFV genome copy number as determined by real time qPCR. RNA was extracted 3 days post-bloodmeal from mosquitoes infected with SFV4(3H)-*FFLuc*-Egf1.0F or SFV4(3H)-*FFLuc*-Egf1.0R was quantified using SFV4 nsP3 primers. Viral genome RNA levels from 10 mosquitoes per virus are shown. Values at 0 represent uninfected mosquitoes. Horizontal bar indicates average genome copy number from infected mosquitoes. This experiment was repeated three independent times showing similar results.

Discussion

Taken together, these results suggest that inhibiting PO activity shuts down the melanisation cascade and provides SFV an advantage to spread in mosquito cell culture. PO has a central and essential role in the melanisation cascade in mosquitoes. As was mentioned before, recent studies suggest that the PO family has expanded in mosquitoes as compared to other members of the Diptera family, such as flies. *Anopheles gambiae* has nine PPOs, whereas *Ae. aegypti* has ten and *C. quinquefasciatus* has nine (Waterhouse et al., 2007) (Arensburger et al., 2010). One can argue that the expansion of the PO family members is due to the pressure of parasitic infections, however neither *Ae. aegypti* nor *Culex quinquefasciatus* act as major parasite vectors. The essential role played by PO in melanisation implies that, by inhibiting this pathway, the end products such as melanin as well as cytotoxic products are not produced. Parasitic wasps with the help of polydnaviruses have taken advantage of this by expression of the PO inhibitor Egf1.0. This protein expressed here by SFV through a duplicated subgenomic promoter resulted in strong inhibition of PO which might be difficult to achieve by just silencing the PO cascade through RNAi. Expressing Egf1.0 from a second subgenomic promoter also ensures a higher production of the protein. Egf1.0 has been shown to keep its inhibitory properties across several insect families (Beck and Strand, 2007) and the results suggest that, expressed in a recombinant manner in SFV, it is successfully exported from the cell and keeps its activity. It was important to confirm that the recombinant Egf1.0 expressed by SFV was exported into the U4.4 culture medium, thus being able to access and inhibit the PO cascade proteins. Inhibition of the PO cascade was not rescued by the addition of PO cascade activators such as *E. coli*. It was also proved that the virus expressing Egf1.0 had no replication or spreading advantage in systems in which melanisation does not exist, such as vertebrate cells.

In the presence of a PO cascade inhibitor, SFV spread through U4.4 cell culture does not increase as extensively as similar experiments with SFV expressing an RNAi inhibitor, however, the effects are still significant for infection dynamics. RNAi probably acts as the major antiviral pathway in mosquitoes; however melanisation clearly has some importance. The next step is to investigate where melanisation acts on SFV in mosquitoes, however for reasons of time this could not be addressed during this project.

Summary of findings

- The melanisation inhibitor Egf1.0 was cloned into SFV4 to be expressed under control of a secondary subgenomic promoter.
- Egf1.0 can inhibit melanisation across several insect species *in vivo* and *in vitro*, including mosquito cell culture medium (U4.4 cells).
- Egf1.0 expressed from SFV4 is exported into the cell culture medium in U4.4 cells.
- Egf1.0 expression increases spread of SFV in U4.4 cell culture medium.
- Egf1.0 expressed by SFV4 can inhibit PO activity and melanisation in U4.4 cell culture medium medium.
- Egf1.0 expressed by SFV4 construct does not confer replication or spreading advantages to SFV in mammalian cell culture medium (BHK-21).
- Inhibition of melanisation in SFV infected mosquitoes decreases insect survival.
- Egf1.0 expression by SFV4 results in enhancement of replication and infectivity in *Ae. aegypti* mosquitoes.

Chapter 6. The role of serine protease inhibitors in controlling the melanisation cascade induced SFV infection

INTRODUCTION

Serine proteases play essential roles in a wide range of biological processes, including innate immunity. In vertebrates they are components of the complement cascade, whereas in arthropods they form part of the Toll and melanisation cascades. Melanisation is involved in wound healing and as a defence mechanism against bacteria, fungi and parasites in insects (Cerenius and Söderhäll, 2004). In this study melanisation has also been identified as an antiviral mechanism in mosquitoes. Once pathogens activate detection systems (PPRs), serine protease cascades are able to amplify and enhance those signals to the appropriate immune response. Regulation of these cascades is complex (Kanost, Jiang, and Yu, 2004). In some cases such as the melanisation pathway, the immune responses regulated by serine protease cascades are cytotoxic and can damage the host. Therefore, the serine protease cascades that lead to melanisation are tightly controlled by serine protease inhibitors called serpins. These proteins ensure that the cascade end products are activated at the proper time and location and therefore it is important to understand how serpins regulate this cascade as well to determine if they regulate anti-viral PO activity.

In the case of melanisation, the protease cascade is orchestrated by clip-domain serine proteases (cSPs). PO, the key enzyme in this reaction, occurs as an inactive precursor PPO. The proteolytic activation of PPO into PO is carried out by phenoloxidase-activating proteases (PAPs) which have been previously activated by cSPs themselves (Soderhall, Cerenius, and Johansson, 1994). cSPs are trypsin-like proteases with an N-terminal disulphide-knotted clip domain and a C-terminal domain with protease activity, whereas PAPs have an N-terminal clip domain and a non-catalytic protease-like domain. Active PAPs have been reported to be very efficient drivers of melanisation (Cerenius and Söderhäll, 2004). Spontaneous melanisation, which is harmful for the host, is inhibited by serine protease inhibitors (serpins) present in the insect's haemolymph (Kanost, 1999). The absence of serpins either through RNAi silencing or mutation results in spontaneous melanisation and formation of melanotic pseudotumors in the trachea, cuticle and internal organs (De Gregorio et al., 2002; Ligoxygakis et al., 2002; Tang et al., 2008; Zou et al., 2008b).

Serpins belong to a super family of proteins with a distinctive structure containing three β-sheets and seven to nine α-helices. The protein folds into a conserved tertiary structure with a reactive centre loop (RCL) near the c-terminal (Law et al., 2006).

In recent studies, using the arthropod model organism *D. melanogaster*, the specifics of serine proteases and serpin interactions regulating the Toll and melanisation cascades have been untangled (De Gregorio et al., 2002; Ligoxygakis et al., 2002). Microbial-induced melanisation in drosophila is controlled by Spn27A (De Gregorio et al., 2002). In the case of the silkworm *Bombyx mori*, 34 serpins have also been described, with serpin-3, a homologue of drosophila Spn27A, controlling microbial induced melanisation (Zou et al., 2009). The tobacco hornworm *M. sexta*, is perhaps the model organism in which the role of serpins regulating the melanisation pathway has been described in the most detail (Fig.6.2).

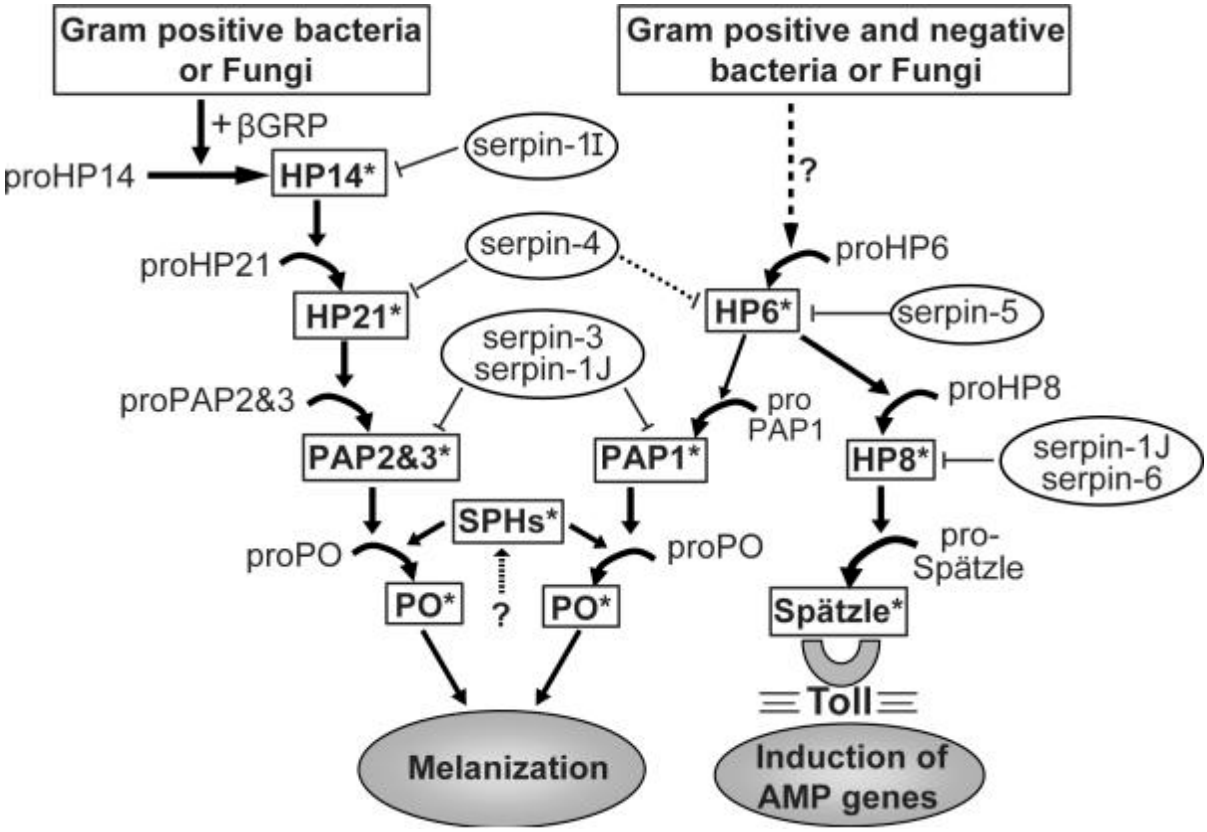

Fig. 6.2. Serpin regulation of the melanisation and Toll pathways in the *M. sexta* model. Haemolymph proteinases (HPs) are also known as cSPs. Dotted lines represent weak inhibition. Adapted from (An and Kanost, 2010).

So far 7 serpins have been identified in *M. sexta*. Serpin-1J and serpin-3 regulate the PO cascade by inhibiting PAPs (Jiang et al., 2003; Zhu et al., 2003). Interestingly, serpin-3 is a homologue of drosophila Serpin27A and *B. mori* serpin-3. Serpins 4 and 5 inhibit melanisation upstream of PAPs (Tong, Jiang, and Kanost, 2005; Tong and Kanost, 2005). In

addition, these two serpins, together with serpin-6, interact with haemolymph proteinases (HP6-8), which are involved in production of PO as well as in the induction of antimicrobial peptide genes that are end products of the Toll pathway (An et al., 2009).

As with most molecular pathways, the information in mosquitoes is much less detailed. Most studies have been done in *A. gambiae* and in *Ae. aegypti*. In *A. gambiae,* 18 serpins have been identified (Suwanchaichinda and Kanost, 2009). Some of them have been shown to be immune regulators of the melanisation cascade triggered by parasite infection, such as SRPN10 and SRPN6, which are expressed by haemocytes and midgut cells and are induced upon *Plasmodium berghei* infection (Abraham et al., 2005; Danielli, Kafatos, and Loukeris, 2003). However, SRPN2 seems to be the major regulator of melanisation upon microbial infection. A knockdown of SRPN2, results in a 97% decrease of *P. berghei* oocyst formation due to an increase in melanisation (Michel et al., 2005). In *Ae. aegypti,* 26 serpins have been identified, 13 of which are induced during microbial infection in adult females (Waterhouse et al., 2007; Zou et al., 2008b). Melanisation in *Ae. aegypti* mosquitoes occurs in two distinct activation pathways; tissue specific and haemolymph. These two pathways are driven by different sets of cSPs and their respective serpins (Zou et al., 2010). Tissue melanisation is characterised by the formation of melanotic pseudo-tumours and is regulated by Serpin-2. This melanisation pathway does not seem to form part of the defence against fungi or parasites, however, it is also involved in the regulation of the Toll pathway (Zou et al., 2010). Haemolymph specific melanisation is controlled by Serpin-1 and it has been associated with parasite defence (Zou et al., 2010). Interestingly, all the serpins characterised as regulators of melanisation during antimicrobial infection are related to each other. The closest homologue of *Ae. aegypti* Serpin-2 is *A. gambiae* Serpin-2. These genes are closely related and in the same gene cluster with *Ae. aegypti* Serpin-1, *A. gambiae* Serpin-1, *D. melanogaster* Serpin27A, *M. sexta* Serpin-3 and *B. mori* Serpin-3 (Kanost, 1999; Suwanchaichinda and Kanost, 2009; Zou et al., 2009; Zou et al., 2010).

Ae. albopictus is an important arbovirus vector, however, due to the lack of genomic studies published to the date, most of its serpins remain uncharacterised. Using the data gathered from the transcriptomic studies in SFV infected *Ae. albopictus* U4.4 cell line (Chapter 3), serpins will be identified and characterised. Also, the possible role that serpins have in controlling SFV infection of U4.4 cells will be investigated.

Chapter 6 The role of serpins in controlling the melanisation cascade in U4.4 cell conditioned medium

Objectives

-Retrieving the sequence of *Ae. albopictus* serpins from transcriptomics data.

-Comparison of *Ae. albopictus* serpin sequences to other insects serpins.

-Characterisation of serpins in U4.4 cells.

RESULTS

Identification of serpins from the transcriptome of *Ae. albopictus* derived U4.4 cells.

Ae. albopictus serpin sequences were retrieved as queries from a BLAST search of the database generated after sequencing RNA of U4.4 cells (infected or non-infected with SFV)(see Chapter 3). A cut off e-value of 0.1 was used. The reference genome alignment used was *Ae. aegypti* as there is no *Ae*. *albopictus* sequenced genome available at present. According to the combined list of accession numbers, nucleotide sequences of putative serpins were retrieved. In most cases the contigs did not cover the whole mRNA sequenced so they were aligned and assembled. In case of discrepancies, the quality of the sequence was retrieved and assessed to correct base errors. Later on the quality of the alignments was confirmed by sequencing cDNA. Serpin sequences were aligned using ClustalX 2.0, a Blosum 30 matrix, with a gap penalty of 10 and an extension gap penalty of 0.005 was selected for the multiple sequence aligment as described in (Suwanchaichinda and Kanost, 2009; Zou et al., 2009). Phylogenetic trees were created with MEGA 5.05 (MegaSoftware) from multiple sequence alignments made in ClustalX.

A total of 11 different serpin transcripts were isolated from the sequences, however, after alignment and corrections only 7 serpins were brought forward for further research. The serpins were named serpin A-K for further analysis. These names are provisional, to avoid confusion with the already characterised serpins of other insect species. The whole available coding transcript sequence was used for the alignments, even if the sequence was incomplete. Parameters were fixed to grant a low penalty for gaps and an extremely low penalty for extension gap (gap penalty: 10, gap extension penalty: 0.005). It should also be noted that sequences for the serpins were retrieved from infected and uninfected U4.4 cells. There were no serpins uniquely expressed in one condition alone. This may be due to low transcription levels.

Serpin alignment and phylogenetic tree

Ae. albopictus serpin sequences were compared after alignment. The relationships between the different serpins are shown below in a multiple sequence alignment. Most serpins share at least 70% of sequence similarity (Fig. 6.1).

```
Aedes_alb_SpnF AGATGCCAATCTGATGATCATCAATACTCTTACATTGAATGCTTCCTGGGAGTATCCACT
Aedes_alb_SpnH AGACGCCAATCTGATGATTATCAACACTGTTACGCTGAACGCTTCCTGGGAACACCCGTT
Aedes_alb_SpnA TATGACGCGTTTGGTGCTGGTCAATGCAATTCATTTCAAGGGCACATGGACGCATCAGTT
Aedes_alb_SpnB TATGACGCGTTTGGTGCTGGTCAATGCAATTCATTTCAAGGGCACATGGACGCATCAGTT
Aedes_alb_SpnG GCAGACCAAACTGGCCATTGCCAATGCTGCCTACTTCAAGGGAACTTGGCAGGCTGAATT
Aedes_alb_SpnD AGCGGTTA---TCACGTTGATCAACGCCATATACTTCAAGGGACTGTGGACTTACCCATT
Aedes_alb_SpnE AGCGGTTA---TCACGTTGATCAACGCCATATACTTCAAGGGACTGTGGACTTACCCATT
Aedes_alb_SpnJ TACCGTCC---TGTTACTGGTGAATGCCATTTACTTCAAGGGACTATGGGTCAACGTTTT
Aedes_alb_SpnC ----------------TGGCCAGNACGGTGTACTACAAGGCGAAGTGGCAAACCATGTT
Aedes_alb_SpnI CACCATGC---TTGTCATTGCCAACACCTTGTATCTGAAGGCTGAATGGGCGGAGCCCTT
Aedes_alb_SpnK GGACATGATGATGCTCCTAGTGAACGCAATCTACTTCAAGGGTACCTGGTTGTATAAATT

Aedes_alb_SpnF GC---TGGGCG-----TACATAAGGATGAATTCCGTTTCCGA----AACGGAGTAAAAGA
Aedes_alb_SpnH TTATACGAGCAGTT--TGCAGAAAAGTGACTTTCAGTTCCTG----AACGGAGTCAGAAA
Aedes_alb_SpnA CAACCCGGCGAGC---ACCAGACCGATGCCGTTCTGGATCAGTGAGACAGAATCAGTGGA
Aedes_alb_SpnB CAACCCGGCGAGC---ACCAGACCGATGCCGTTCTGGATCAGTGAGACAGAATCAGTGGA
Aedes_alb_SpnG CAAGCCAGATCAA---ACCAACAAGGAAATCTTCTACGTTTCCAGTGAACGCCAAGCGTT
Aedes_alb_SpnD CCCA--GAATACA---CGCCAATGTTGACCTTCCATGGCAAG----CAGAAGCAAGTGCA
Aedes_alb_SpnE CCCA--GAATACA---CGCCAATGTTGACCTTCCATGGCAAG----CAGAAGCAAGTGCA
Aedes_alb_SpnJ CCTACCAAGTGCT---ACCACAGAGCAACCGTTCACTACCGCATTTCGTAAAACCGTTCA
Aedes_alb_SpnC CATCGAACAGGAC---ACCCGACCAAGACCGTTCCATATCAACGGACCTAATGCTGCTCC
Aedes_alb_SpnI CCTGAGAGATGGA---ACCAAGCCGAGACCATTTTTCCCGGATGGGCCCAACAGACCGTC
Aedes_alb_SpnK CAACGAAACGGAAACAAACAAACGGGCTACATTCGAATCTTCCAAAAATAATAAGATGCC

Aedes_alb_SpnF AGTTGACATG------ATGCAAATTAACAAGGGACTCAGATCTTGTGAAATTG--GCGAT
Aedes_alb_SpnH AGTTGATATG------ATGCGAACTTACAAGGGCTTTCGGTATTGTGAAATCG--ATGCT
Aedes_alb_SpnA TGTTCCCATG------ATGAACACCAAAAAACACTTCAAGCACGGTGTTTTCG--ATGAC
Aedes_alb_SpnB TGTTCCCATG------ATGAACACCAAAAAACACTTCAAGCACGGTGTTTTCG--ATGAC
Aedes_alb_SpnG CGTCGACATG------ATGCATGTCTTGGGAACCTTCAACCACGCTGCTAACG--AGAAG
Aedes_alb_SpnD AGCTCCATTC------ATGGAACAAAATGGTCAGTTCTACTACGATGATTCAG--CGGCA
Aedes_alb_SpnE AGCTCCATTC------ATGGAACAAAATGGTCAGTTCTACTACGATGATTCAG--CGGCA
Aedes_alb_SpnJ AGTCCCATTC------ATGAAGCAAATTCAGGAACACTACTTCGTTGATTCCA--AAGTA
Aedes_alb_SpnC AGTTGACATCGATACTATGGCCACTAATGGGTGTTTTCCGACCTACGAAGATA--AACAA
Aedes_alb_SpnI GATCAACGTTCCGATGATGGTTCATGGTGGATGTTTCCCGTACTATTACTGGA--AGGAA
Aedes_alb_SpnK GGTCCATATGATGTCGCAGACGAACAAACTTCGCTTCGGTGAAATCAACTATGGCATGTA
```

Fig. 6.3. Multiple nucleotide sequence alignment of *Ae. albopictus* serpins. The central region of the serpins, where complete sequences were available was used for simplicity to visualise the relative homology between the transcripts.

```
Aedes_alb_SpnA VNFSDNTAAAKKINTWVEQKT-NDKIKDLISPDS--------LDDMTRLVLVNAIHFKGT
Aedes_alb_SpnB VNFSDNTAAAKKINTWVEQKT-NDKIKDLISPDS--------LDDMTRLVLVNAIHFKGT
Aedes_alb_SpnD SSFLQPEKCRRTDNNWVNKTT-HGRISELVTPDG--------LEGAV-ITLINAIYFKGL
Aedes_alb_SpnE -PFSNPKNAAELINNWVNKTT-HGRISELVTPDG--------LEGAV-ITLINAIYFKGL
Aedes_alb_SpnF VPISGPEQVVNSVXRWASRFT-NGLVGDVFYGGG--------YSRDANLMIINTLTLNAS
Aedes_alb_SpnH VPISAVRNRW-ILSTDGLPVSPTGWXGDVFNGAG--------YSRDANLMIINTVTLNAS
Aedes_alb_SpnC ----------------------------------------------AXTVYYKAK
Aedes_alb_SpnG LDFEKDADGQRLYINNWVENVTQGEIKDLLIPGS--------ITKQTKLAIANAAYFKGT
Aedes_alb_SpnJ QR-LPANRKGC-DNQ-LGFDSTRGMIPQLVRPEN--------IKDTV-LLLVNAIYFKGL
Aedes_alb_SpnK SKGPRIKDSFP-CSKAQQH-NERC-LCKCIPGCK-C-RMG-HPNPRKYSTGYNRTGYLSG
Aedes_alb_SpnI SRMIRPKM-PSLGGSLTSATTTTTKMKTIPRRSQRPSNEANGTPKNRLKSTWAMEFLQSL


Aedes_alb_SpnA WTHQFNPASTRPMPFWI--SETESVDVPMMNTKKHFKHGVFDDLGLAALEMTYND----S
Aedes_alb_SpnB WTHQFNPASTRPMPFWI--SETESVDVPMMNTKKHFKHGVFDDLGLAALEMTYND----S
Aedes_alb_SpnD WTYPFP--EYTPMLTFH--GKQKQVQAPFMEQNGQFYYDDSAALDAQLXRLSYRG----G
Aedes_alb_SpnE WTYPFP--EYTPMLTFH--GKQKQVQAPFMEQNGQFYYDDSAALDAQLLRLSYRG----G
Aedes_alb_SpnF WEYPLLG--VHKDEFRF--RNG-VKEVDMMQINKGLRSCEIGDL--RIVELAYER----T
Aedes_alb_SpnH WEHPFYTSSLQKSDFQF--LNG-VRKVDMMRTYKGFRYCEIDAL--RIVELAYKR----T
Aedes_alb_SpnC WQTMFIEQDTRPRPFHINGPNAAPVDIDTMATNGCFPTYEDKQLDAKIVGLPYQK----D
Aedes_alb_SpnG WQAEFKPDQTNKEIFYVS--SERQAFVDMMHVLGTFNHAANEKLGCHVLEMPYKGQDEST
Aedes_alb_SpnJ WVNVFLPSATTEQPFTT--AFRKTVQVPFMKQIQEHYFVDSKVLKAQLVRLPYSD----G
Aedes_alb_SpnK HDDAPSERNLLQGYLVV-IQRNGNKQTGYIRIFQK--DAGPYDVADEQTSLR-NQLWHVL
Aedes_alb_SpnI VESSITSTNTSPKKSMVLKPNTWTLKIEHKTPFAPLTIGLNGKLAGRLQKSCRN----AP


Aedes_alb_SpnA DVSMLILLPHERT-----------GLTKLEENLQNID---------IPDMLTKMYSQEVE
Aedes_alb_SpnB DVSMLILLPHERT-----------GLTKLEENLQNID---------IPDMLTKMYSQEVE
Aedes_alb_SpnD KFAMYFILPHQGK-----------TVDDVLEKITPTT---------LHQALWYMDETEVN
Aedes_alb_SpnE KFAMYFILPHQGK-----------TVDDVLEKITPTT---------LHQALWYMDETEVN
Aedes_alb_SpnF SDLSMLFIKSDSQ-----------PLEKVVERLDLQM---------YRTI----------
Aedes_alb_SpnH ADLSMLIIKSDSQ-----------PLEKVVERLDLGM---------YRSIDERLYEDRFK
Aedes_alb_SpnC KSTMYX-------------------------------------------------------
Aedes_alb_SpnG RISMFVFLPPAAPN----------SLDKVLARLTSDTGI-------LSEIVNEGIPRMVD
Aedes_alb_SpnJ RFSMIIVLPNETAV-ASS-T-SHLILSMQLSETWKRSK--------TFNCRDSALITIVP
Aedes_alb_SpnK RLGARIALGRATV--------WR-AVDDCSSPKDPVPIG-----RNGAPCQRKPLPGNFP
Aedes_alb_SpnI TPCLSLPTPCI-RLNGRSPS-EMEPSRDHFSRMGPTDRRSTFR-WFMVDVSRTITGRKCK
```

Fig. 6.4. Multiple amino acid sequence alignment of *Ae. albopictus* serpins. The central region of the serpins, where complete sequences were available was used for simplicity to visualise the relative homology between the transcripts.

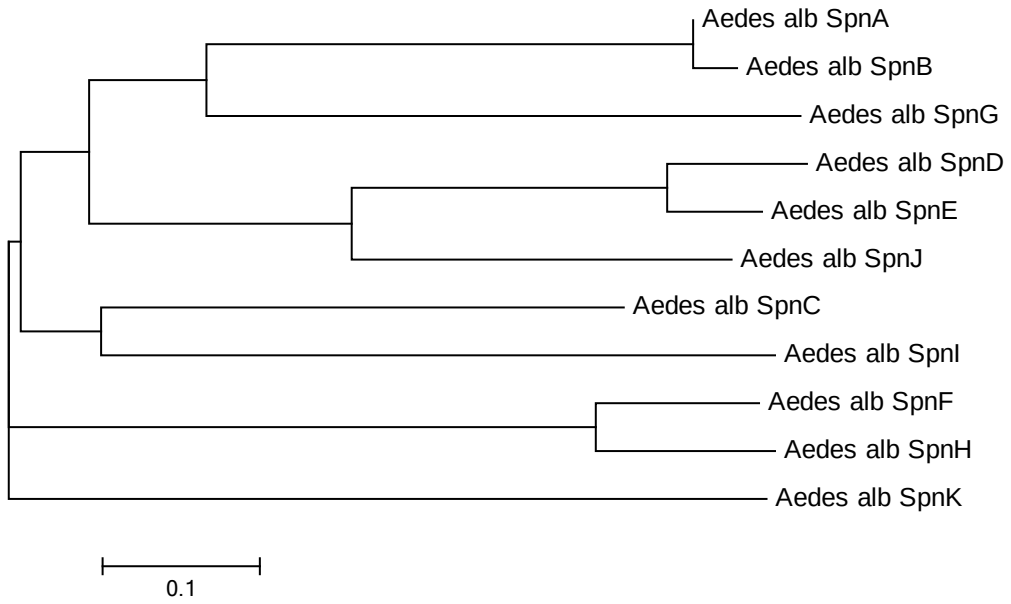

Figure 6.4. Phylogenetic tree of *Ae. albopictus* serpins found in the U4.4 transcriptome. The un-rooted tree is based on a multiple sequence nucleotide alignment created in ClustalX with previously described parameters. Serpins A and B, D and E and F and H are the most closely related. Serpin K is un-rooted

Identification of *Ae. albopictus* serpin homologous to other insect serpins

In order to easily describe the relationships within the phylogenetic tree, each clade of related serpins was colour coded. This colour clade classification is based on previous phylogenetic studies on serpins (Zou et al., 2009) (Fig.6.4).

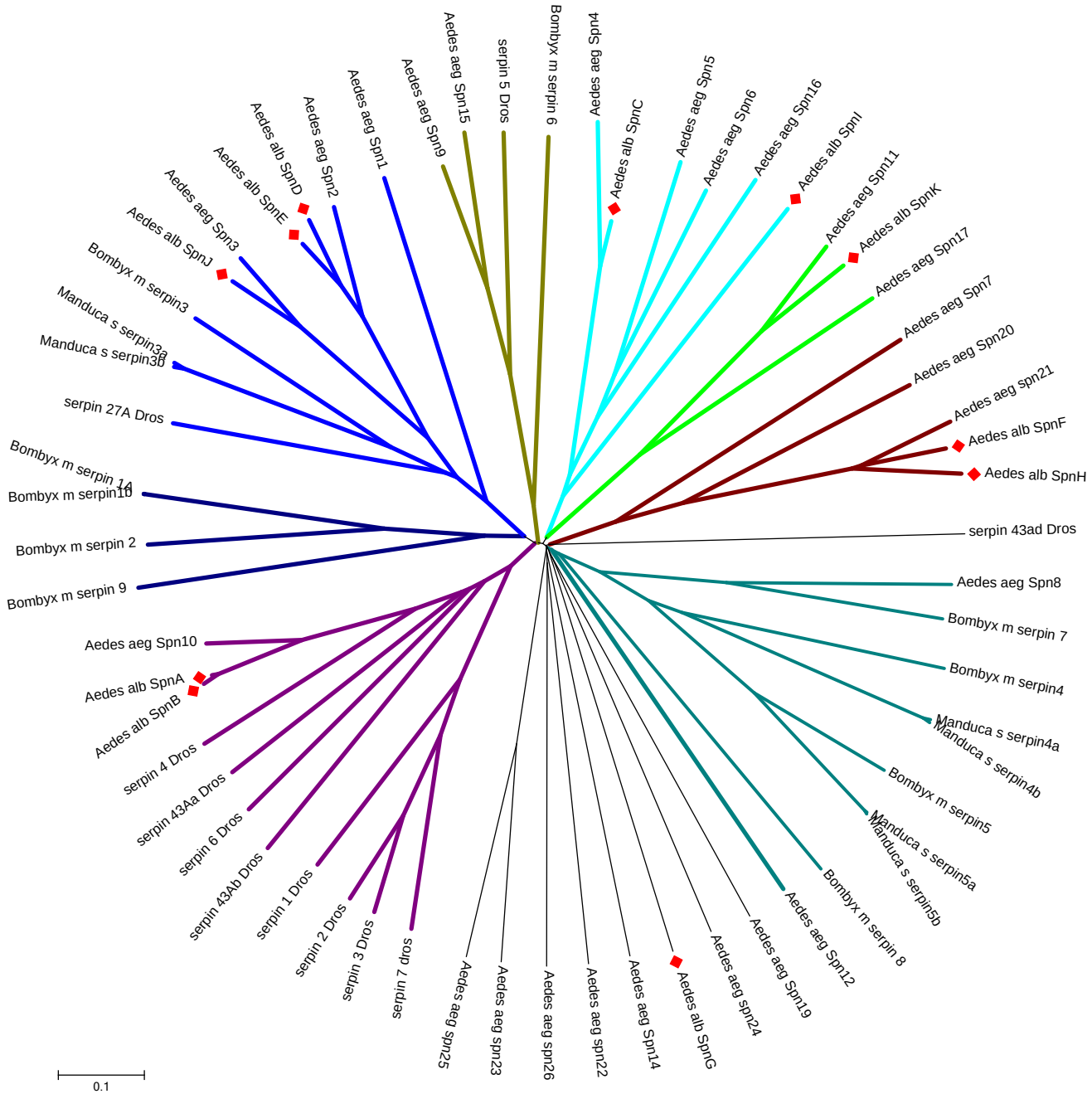

Fig.6.4. Phylogenetic relationship between the serpins of *Ae. albopictus*, *B. mori*, *M. sexta*, *D. melanogaster* and *Ae. aegypti*. Red diamonds indicate the *Ae. albopictus* serpins identified in this study. Based on alignment similarities serpins were classified in several coloured groups. Unclassified serpins are un-rooted.

The dark yellow clade is formed by *B. mori* serpin 6, *D. melanogaster* Serpin-5 and *Ae. aegypti* serpin 9. They have been linked to *M. sexta* serpin-6, which is known to form complexes with HP8 and PAP1/PAP3 (Wang and Jiang, 2004). However, no *Ae. albopictus* serpins were found in this clade.

The brown clade is represented by *Ae. aegypti* serpins-7, 20 and 21, however, there is little information about the serpins in this clade. The closest homologues to serpins-20 and 21 are *C. quinquefasciatus* serpins. In one study, the depletion of *Ae. aegypti* serpin-20 does not activate the PO cascade (Zou et al., 2010). There is no molecular analysis or functional characterisation of serpins-7 and 21. Serpin H and F are in this clade.

The green clade is formed by *Ae. aegypti* serpins-11 and 17. Depletion of either of these two serpins does not activate the PO cascade, therefore they are not involved in the melanisation reaction (Zou et al., 2010). No other experimental data is available for these serpins. *Ae. albopictus* serpin K also belongs to this clade.

The cyan clade is formed by *Ae. aegypti* serpins-4, 5, 6 and 16 which have been classified as homologues of *A. gambiae* serpins-4, 5, 6 and 16 (not used in this study due to simplicity) (Zou et al., 2009). This group of serpins shows a greater expansion in mosquitoes than in other insects, although no biochemical or characterisation analysis has been carried out for any of the clade members with the exception of the *A. gambiae* homologue of serpin-6 which has been linked to mediating anti-malaria immunity in the mosquito (Abraham et al., 2005; Zou et al., 2009). *Ae. albopictus* serpins C and I belong to this clade.

The aquamarine clade is formed by *B. mori* serpins-4, 7 and 8, *M. sexta* serpins-4s, 5s and *Ae. aegypti* serpin-8 and 12 belong to this clade. In previous classification and characterisations these serpins have been found to be related (Zou et al., 2009). Biochemical studies with some members of this group such as *M. sexta* serpin-4 and 5 suggest that these serpins can inhibit multiple humoral proteases involved in immune responses (Tong, Jiang, and Kanost, 2005). No *Ae. albopictus* serpin homologues were found in this clade.

The blue clade is formed by *D. melanogaster* serpin-27A, *M. sexta* serpin-3, *B. mori* serpin-3 and *Ae. aegypti* serpins-1, 2 and 3. *A. gambiae* serpins-1, 2 and 3 are also part of this group but were not included in this analysis (Suwanchaichinda and Kanost, 2009). Serpins in this

group are known to regulate the PO cascade as well as Spatzle activation (Abraham et al., 2005; Hashimoto et al., 2003; Zhu et al., 2003; Zou et al., 2010). The *Ae. albopictus* serpins in this clade are D, E and J.

The dark blue clade contains *B. mori* Serpin-1A, 1B, 2 and 9. *B. mori* serpin-2 (Bmserpin-2) is known to play an antiviral role in BmNPV infection in the silkworm (Pan et al., 2009). It has been found that *B. mori* serpin-2 negative mutants allow lower levels of viral replication. The current mechanism of this response has not been clarified, although it has been suggested that the serine proteases inhibited by Bmserpin-2 can cleave viral proteins. These proteases remained unidentified and the link between Bmserpin-2, its target serpin protease and melanisation were not investigated in this study.

The purple clade is formed by drosophila serpins-1, 2, 3, 4, 6, 7, 43a, 43b and *Ae. aegypti* serpin-10. These drosophila genes form part of a cluster which is involved in regulating the Toll pathway and has been extensively described (Hoffmann and Reichhart, 2002). These genes are in the drosophila's second chromosome 43A–E region (hence their names) and mutations in these proteins result in black necrotic spots, which is another name by which these genes are known (*nec*) (Heitzler et al., 1993). These black spots in 43A-E mutant flies are due to the accumulation of cleaved Spatzle and constitutively active serine proteases in the Toll pathway (Levashina et al., 1999). The presence of black necrotic spots could also be due to a cross-talk between the melanisation and Toll pathways. However, in drosophila these two pathways have not been linked with this cluster of serpins. There is little information about the rest of Drosophila serpins in this group. Most of them are linked to signalling in olfactory, neurological, hormonal and developing functions (Osterwalder et al., 2004; Reichhart, 2005; Smart et al., 2008). *Ae. aegypti* serpin-10 was discovered in a transcriptome from uninfected mosquito salivary glands, however, its functions remain unknown (Valenzuela et al., 2002). Two *Ae. albopictus* serpins were found in this clade, serpin-A and serpin-B.

There are several serpins that do not cluster into any clade. These serpins are not homologous to any other serpins used in the analysis; however, they are still classified as serpins due to their sequence characteristics.

Serpins-A and B are likely to be homologues of *Ae. aegypti* serpin-10. Serpin-C is homologous to *Ae. aegypti* serpin-4. Serpins-D and E are closely related to *Ae. aegypti* serpin

2 and, in the same clade, serpin-J is likely to be homologous to *Ae. aegypti* serpin-3. Serpins F and H are relatives of *Ae. aegypti* serpin-21 and to a lesser extent serpin-20. Serpin-K is a homologue of *Ae. aegypti* serpin-11. Serpin-I could be a homologue of either *Ae. aegypti* serpins-5, 6 or 16. Serpin G does not have any close homology to any other serpins used in this analysis.

These relationships are based purely on sequence homology. It has to be noted that the same homology analysis were done on the amino acid sequences. However, nucleotide alignments are shown since they provide higher homology resolution. In order to fully classify them biochemical and functional studies need to be carried out.

Silencing of serpins and effect on PO activity

In order to further characterise and investigate the role of *Ae. albopictus* serpins in melanisation, serpins were silenced using specific long dsRNA in the following manner: serpin sequences which will produce long dsRNA fragments of at least 400 bp were selected and checked for excessive sequence homology before designing primers, those were designed to target specific serpins. RNA was extracted from U4.4 cells and reverse transcription was done. Initially, unique serpin primers containing a T7 promoter region were used to amplify fragments of at least 400 bp by PCR. This strategy was not successful for most of the serpins since their transcripts are not very abundant and the addition of the T7 promoter to the primer made the PCR reaction less efficient. Therefore, external primers without the T7 promoter were designed and DNA fragments were amplified. Using these fragments in addition to a second set of internal primers containing the required T7 promoter site, DNA fragments were successfully generated. From these internal DNA fragments flanked by T7 sequences, long dsRNA were generated as stated in Materials and Methods (Chapter 2). Long dsRNA molecules were then transfected into U4.4 cells and PO activity was measured from the cell culture medium after 48 h using the PO activity assay described Materials and Methods (Chapter 2). Each of the identified *Ae. albopictus* serpins was named with a letter A-K until further characterisations were made.

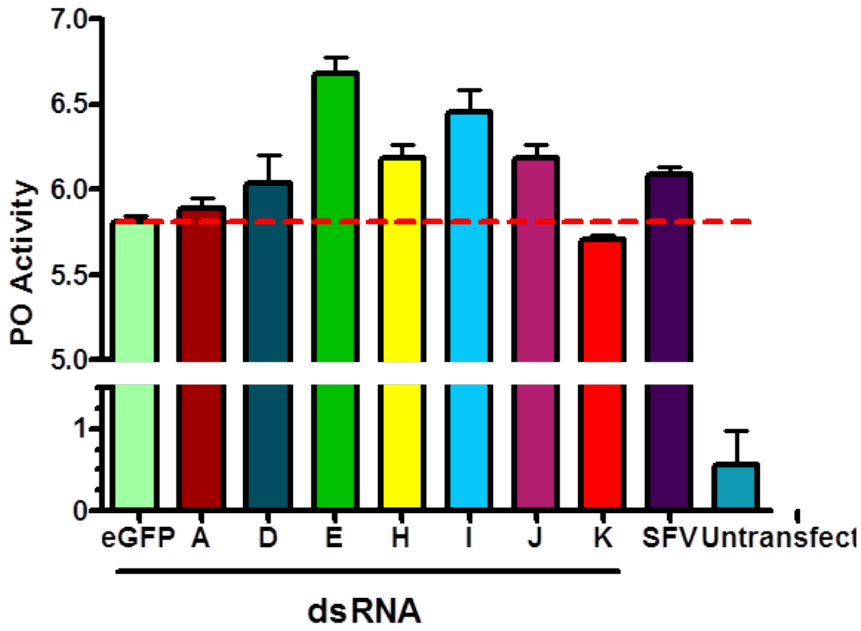

Fig. 6.6. PO activity assay in U4.4 cell culture medium after serpin silencing. 6.5 x 10^5 cells U4.4 cells were transfected with specific serpins long dsRNAs and PO activity was measured from the cell culture medium after 48 h using the PO activity assay The red discontinuous line represents the average of the unspecific long dsRNA control. Purified SFV is used as a positive control and cell culture medium from untransfected cells is used as a negative control. Each of the *Ae. albopictus* silenced serpins was identified with a letter A-K. One unit of PO activity was defined as ΔA490=0.001 after 30 minute incubation; absorbance changes due to oxidation of the dopamine substrate (see Materials and Methods) by PO activity. The Y axis indicates PO activity units (U). Each bar represents the mean from 10 reactions; error bars represent standard deviation. This graph is representative of 3 independent experiments.

As expected, the addition of purified SFV to conditioned cell culture medium also resulted in an increase of PO activity. Silencing of serpins E, H, I and J resulted in a significant increase of PO activity and, therefore, these serpins appear to inhibit serine proteases directly involved in this pathway in *Ae. albopictus* (serpin E, t-test: P<0.0001; serpin H, t-test: P=0.0003; serpin I, t-test: P=0.0002; serpin J, t-test: P=0.0003). There was also an increase with serpin D, although it was not significant. These serpins are homologues of *Ae. aegypti* serpins 2, 3 and 21. All these genes have been previously described as being directly involved in controlling the PO cascade (Valenzuela et al., 2002; Zou et al., 2010). It has to be noticed

that the transfection of unspecific (eGFP) long dsRNA also triggers PO activity, probably due to the nature of the Lipofectamine 2000 liposomes which are able to trigger the PO cascade. Indeed amphipathic molecules and alcohol activate insect PPOs (Kanost, 2008). Therefore, only serpins that produced a higher than control increase of PO activity were considered for further studies.

Silencing of serpins and effect on SFV replication and spread

The effect that serpins have on SFV infection and spread in U4.4 cell culture was examined in the following experiment. Serpins were silenced using specific long dsRNAs as described above and infected with SFV expressing *RLuc*.

A monolayer of U4.4 cells was transfected using Lipofectamine 2000 with the serpin specific long dsRNA fragments described in the previous experiment. After 24 h, the monolayer of cells was infected with a low MOI (0.005) of SFV4(3H)*RLuc*, thus allowing it to spread through the culture and luciferase readings were taken after 48 h post infection. Long dsRNA from eGFP was transfected as a negative control.

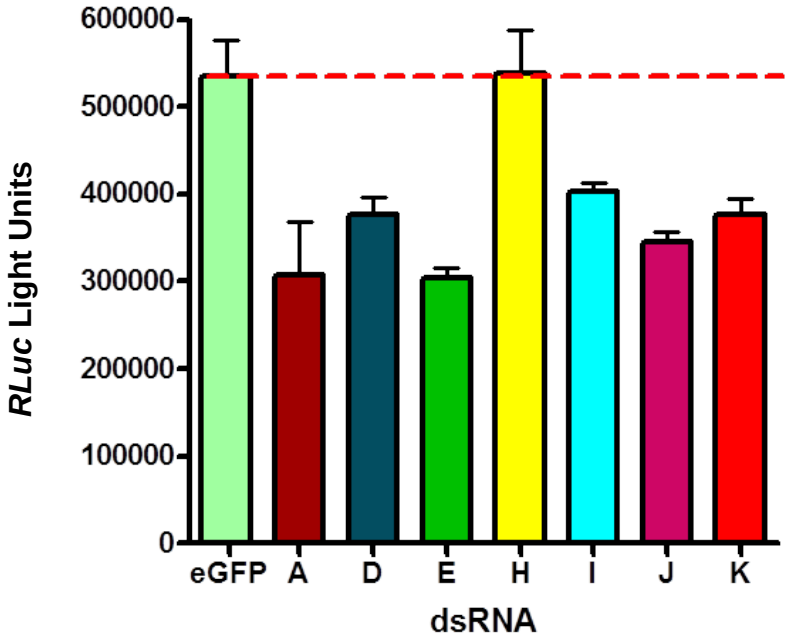

Figure 6.7. The effect of serpin silencing on SFV replication and spread. Long dsRNA from different serpins were transfected into U4.4 cells as shown. The red segmented line represents the average of the control wells transfected with unspecific long dsRNA (eGFP). Each of the *Ae. albopictus* serpins was identified with a letter (A-K). *RLuc* was measured 48 h p.i. Each bar represents the mean from triplicate cultures; error bars represent standard deviation. This experiment is representative of three independent experiments.

All the serpins, with the exception of serpin H, had a significant effect on reducing the amount of viral spread and replication (serpin A, t-test: P=0.0364; serpin D, t-test: P=0.0265; serpin E, t-test: P=0.0059; serpin I, t-test: P=0.0370; serpin J, t-test: P=0.0116; serpin K, t-test: P=0.0255). Serpin H is homologous to *Ae. aegypti* serpin-21 and, as mentioned before, there is no evidence of this serpin's function in the PO cascade. It is a peculiar case that this serpin, even when PO activity is increased, does not reduce viral spread and replication. This serpin could belong to a different pathway that does not affect SFV, or be a transfection artefact. There are no publications in the literature about this serpin and the closest of its relatives, serpin-20, has been shown to not be involved in the activation of PO (Zou et al.,

2010). It should also be highlighted that only dsRNA was transfected, transcript silencing efficiency was not further verified by, for example, RT-PCR.

Silencing of multiple serpins and effect of SFV replication and spread

The serpins from the blue clade, which are involved in melanisation were selected for further analysis. As in previous experiments in this chapter, serpins were silenced using specific long dsRNAs following which the culture was infected with SFV4(3H)RLuc and viral spread and replication were monitored.

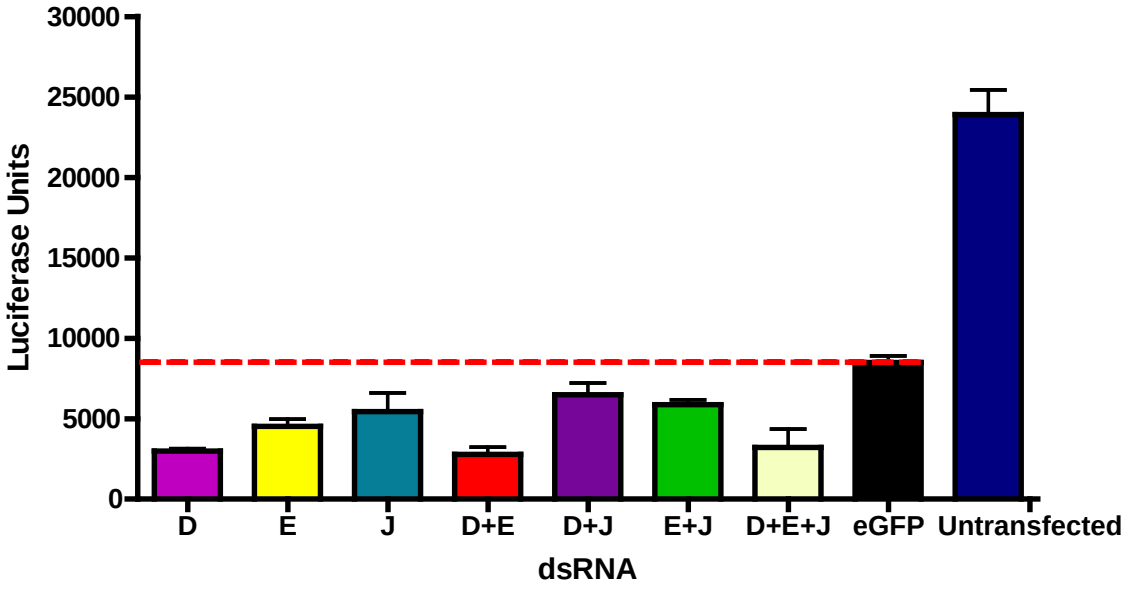

Figure 6.8. The effect of serpin silencing on SFV replication and spread. Long dsRNA from different serpins were co-transfected into U4.4 cells as indicated. If multiple serpins were co-silenced the same fraction of each long dsRNA was used. The red segmented line represents the average of the control wells transfected with unspecific long dsRNA (eGFP). RLuc was measured 48 h p.i. Each bar represents the mean from triplicate cultures; error bars represent standard deviation. This experiment is representative of three independent experiments.

There is no significant difference between silencing these serpins individually or jointly, though they reduced SFV replication and spread compared to eGFP control. This is not surprising as each of the serpins work at different steps within the pathway. Most likely

silencing the serpin at the top of the activating cascade will make the role of the downstream serpins unimportant, or vice versa.

All these experiments are preliminary and only shed some light on the possible functions of the *Ae. albopictus* serpins. It is worth noticing that *Ae. aegypti* serpin-1 is responsible for humoral melanisation, which is thought to affect virus infection. In this study no serpin-1 was retrieved from *Ae. albopictus* derived U.4.4 transcriptome sequencing. However, we cannot completely discount that one of those three serpins found in the blue clade (E, D, and J) are its homologues.

DISCUSSION

In this study, several *Ae. albopictus* serpins were identified and associated with *Ae. aegypti* homologues. Serpins A and B are likely to be homologous to *Ae. aegypti* serpin 10. Serpin C is homologous to *Ae. aegypti* serpin 4. Serpins D and E are closely related to *Ae. aegypti* serpin 2 and, in the same clade, serpin J is likely homologous to *Ae. aegypti* serpin 3. Serpins F and H are relatives of *Ae. aegypti* serpin 21 and, to a lesser extent, to serpin 20. Serpin K is a homologue of *Ae. aegypti* serpin 11. Serpin I could be a homologue of either *Ae. aegypti* serpins 5, 6 or 16. Serpin G does not have any close homology to any other serpins used in this analysis.

Further analysis of these *Ae. albopictus* serpins showed that some, especially those related to *Ae. aegypti* serpin 2, drosophila 27A, *M. sexta* and *B. mori* serpin 3, increase PO activity when silenced, correlating to similar findings in *Ae. aegypti* (Zou et al., 2010). In addition, *Ae. albopictus* Serpin H, or *Ae. aegypti* serpin 21 also induces an increase PO activity when silenced. This fact was previously undocumented. SFV spread and replication in U4.4 cell culture is reduced when all serpins are silenced (with the exception for serpin H), and thus the PO cascade is constitutively activated.

Several issues arose during these experiments. Some of the serpins, such as serpin C, had only been partially sequenced, and the available sequenced information was not enough for the transcript to be silenced. Additionally, some serpin sequences, especially among those in the same clade, are very similar and therefore it was difficult to find optimal primers that could distinguish one from another. All the DNA fragments amplified were sequenced before the production of long dsRNAs in order to remove cross-silencing. For example serpin B was removed as it was very similar to serpin A and the long dsRNA sequence was not specific enough to distinguish between the two. Therefore when silencing serpin A, both serpin A and B were silenced. It was also noted that liposomes, and amphipathic molecules in general, such as Lipofectamine 2000 can trigger melanisation during transfection as has been described for amphipathic molecules (Kanost, 2008).

The PO cascade is a very complex, and the characterisation of the serpins that control it in *Ae. albopictus* is no simple task. This study showed some preliminary data as a pioneering step towards establishing the mechanisms that control this pathway in *Ae. albopictus*.

The better studied PO cascade in *Ae. aegypti* mosquitoes occurs through two different pathways (Zou et al., 2010). It can only be assumed that the same case would occur in *Ae. albopictus* mosquitoes. Characterisation of the specific serpins which control the two distinct melanisation pathways is a difficult assignment since the specific serpins that control each of these pathways (serpin 2 for tissue melanisation and serpin 1 for haemolymph melanisation) are quite similar at both nucleotide and amino acid level. The apparent absence of *Ae. albopictus* serpin-1 from sequencing data prevents the possibility of knowing if this serpin has any importance in *Ae. albopictus* PO response against SFV or if that serpin is one of the three found in the same clade (D, E, and J). As mentioned before, serpin 2 of *A. aegypti* is also a regulator of the Toll pathway (Bian et al., 2005; Zou et al., 2010). The involvement of serpins is this pathway also occurs in drosophila. Serpins 27A, 5, 43AC and 77BA are known to be involved in Toll regulation in the fruit fly (Green et al., 2000; Levashina et al., 1999; Ligoxygakis et al., 2002; Robertson et al., 2003). With regards to our system, the decrease in SFV replication and spread following the knock down of serpin 2-related serpins in *Ae. albopictus* derived U4.4 cells could be due to the increase in Toll activity. However, this statement is unlikely since alphaviruses are not affected by Toll (Fragkoudis et al., 2008c). Therefore, the effect of silencing *Ae. aegypti* serpin 2 homologues on SFV in *Ae. albopictus* cells is likely due to the activation of pathways leading to PO activity.

The importance that melanisation has on pathogens, as well as the efficiency by which serpins control this pathway, is demonstrated by the fact that the insect host is not the only organism expressing serine protease inhibitors. It is known that parasites, such as microfilariae of *Brugia malayi*, express serpins in order to inhibit melanisation to their advantage (Zang et al., 1999). Another example of a serpin-like peptide expressed by an insect pathogen is Egf1.0 protein from the MdBV (Beck and Strand, 2007). However, to date no arboviruses have been shown to express serpins.

Understanding the role of serpins may be of use in controlling mosquito viability when spreading infectious parasitic and viral diseases. The interaction of serpins with cSPs has been correlated with mosquito life expectancy. In *A. gambiae*, depletion of SRPN2 increases adult female mortality late in life (An et al., 2011), thus this gene has been proposed as a novel insecticide target (Read, Lynch, and Thomas, 2009). ‘Late in life’ acting insecticides inhibiting SRPN2 would kill mosquitoes prior to parasite transmission by minimizing selection pressure for resistance. Using the same principle, insecticides against aedine

mosquitoes could be developed which would reduce the number of mosquitoes prior to virus transmission.

Summary of findings

- *Ae. albopictus* serpin sequences were identified from the transcriptome of U4.4 cells analysed in Chapter 3.
- *Ae. albopictus* serpin sequences were aligned and classified alongside serpins from other insect species, finding several potential homologues.
- Silencing of several *Ae. albopictus* serpins triggers the PO cascade in conditioned U4.4 cell culture medium.
- Silencing of some *Ae. albopictus* serpins reduces SFV spread and replication in U4.4 cells culture.

Concluding Remarks

During this project, uncharacterised innate immune responses of *Ae. albopictus* derived U4.4 cells to SFV infection have been identified and investigated. The role of the PO cascade as a novel immune response against arbovirus infection has been determined.
In the transcriptomic study it was found that most cellular transcripts are not differentially regulated during infection. In fact, only 2% of gene transcripts were up-regulated and 4% of gene transcripts were down-regulated. The majority of the up-regulated transcripts induced upon SFV infection of U4.4 cells were related to nucleic acid processing and serine proteases, as well as other immune related genes, unfolded protein response genes and other stress responses. On the other hand, transcripts which were down-regulated upon SFV infection were involved in exocytosis and vesicle export pathways; RNA degradation, and reactive oxygen species metabolism. The results of this transcriptome study are still to be validated by other means such as RT-PCR, however, contig coverage gives a very high quality, quantified number of RNA bases per transcript so PCR verification was not a priority. The next step would have been silencing of up-regulated genes with RNAi. Several candidate genes were going to be silenced and their effect on SFV infection monitored. This was not carried out further for reasons of time, since it was decided to follow up the role of the melanisation pathway in SFV infection in more detail.

Until now, melanisation was not considered to act as an immune response against arboviruses in mosquitoes. In this thesis it has been demonstrated that there is a melanisation cascade and PO activity in U4.4 cell-conditioned medium. The model arbovirus SFV and *E. coli* can activate the PO cascade in conditioned cell culture medium, however, the mechanisms by which SFV triggers PO activity are still unknown. SFV replication and spread is affected n all likelihood by reaction intermediates and cytotoxic by-products of the PO cascade, but how the PO cascade negatively affects the virus infection and spread it is also not known.

The bracovirus melanisation inhibitor Egf1.0 was cloned into SFV4 to be expressed under control of a secondary subgenomic promoter in order to further investigate the PO cascade. Egf1.0 works in an inter species manner, and was found to inhibit the PO cascade in mosquito cell culture (U4.4 cells). As expected, Egf1.0 expressed from a second subgenomic promoter within SFV was exported into the U4.4 cell culture medium. The expression of this

protein was found to increase the spread of SFV. However, in mammalian cell culture (BHK-21 cells), this protein does not confer any replication or spreading advantages to SFV. *In vivo* experiments showed that the inhibition of melanisation by Egf1.0 in SFV infected mosquitoes decreases vector survival. Egf1.0 expression by SFV4 also resulted in the enhancement of viral replication and infectivity in *Ae. aegypti* mosquitoes. The transcriptome of U4.4 cells also allowed the identification of *Ae. albopictus* serpin sequences which were used in further study of the PO cascade. *Ae. albopictus* serpin sequences were aligned and classified alongside serpins from other insect species, finding several potential homologues. Silencing of several of these serpins triggers the PO cascade, reducing SFV spread and replication in conditioned U4.4 cell culture medium.

In summary, my results give the first insight into the transcriptome of *Ae. albopictus* U4.4 cells, and provide much needed, currently missing genomic information for this important vector species. I also show that the PO cascade has antiviral activity and can be added to the list of immune responses responsible for controlling arbovirus replication in mosquitoes.

Future research in the antiviral role of the PO cascade is required. A large number of players in the mosquito melanisation cascade are unknown. One of the most important aspects that it is yet to be determined is the identification of the PAMP receptor that triggers this cascade. These types of receptors have an extracellular soluble particle recognition protein nature and remain largely uncharacterised; however there are several candidate proteins involved, such as immunolectins. The nature of the PAMP that activates the cascade, whether it is a lipid vesicle or glycoproteins, is also unknown and should be investigated in the future. It is suggested that ROS are the cascade's end product responsible for virucidal activity. The interaction between these reactive molecules and the virion are unknown and also deserve further investigation.

It has been shown that in order to mount a successful defence against viral infection, mosquitoes require input from different immune pathways. The magnitude and intrinsic nature of these pathways is such that a lot more research and resources will be needed to understand the mechanisms behind arbovirus defence in mosquitoes. How SFV (and possibly other viruses) activate the PO cascade and how this pathway is regulated should be investigated. The transcriptome data obtained in the course of this study may help these

investigations. This thesis opens the door to further investigations into the role of the PO cascade as an antiviral mechanism.

Mosquitoes are part of nature and play an essential role in ecosystems, specifically by bringing nitrogen back from higher vertebrates into the environment. Removing mosquitoes as a mechanism to reduce human arboviral infections, wheather with pesticides or traps, might just be a short term solution which will damage the environment. Understanding the role of antiviral pathways in mosquitoes might lead, someday, to applications which will help in the fight against arboviral diseases, such as dengue fever, without the need for eliminating mosquitoes, for example with the creation of transgenic mosquitoes lines or the use of symbiotic organisms, such as wolbachia.

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Appendix

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Phenoloxidase Activity Acts as a Mosquito Innate Immune Response against Infection with Semliki Forest Virus

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Abstract

Several components of the mosquito immune system including the RNA interference (RNAi), JAK/STAT, Toll and IMD pathways have previously been implicated in controlling arbovirus infections. In contrast, the role of the phenoloxidase (PO) cascade in mosquito antiviral immunity is unknown. Here we show that conditioned medium from the *Aedes albopictus*-derived U4.4 cell line contains a functional PO cascade, which is activated by the bacterium *Escherichia coli* and the arbovirus Semliki Forest virus (SFV) (*Togaviridae*; *Alphavirus*). Production of recombinant SFV expressing the PO cascade inhibitor Egf1.0 blocked PO activity in U4.4 cell- conditioned medium, which resulted in enhanced spread of SFV. Infection of adult female *Aedes aegypti* by feeding mosquitoes a bloodmeal containing Egf1.0-expressing SFV increased virus replication and mosquito mortality. Collectively, these results suggest the PO cascade of mosquitoes plays an important role in immune defence against arboviruses.

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Introduction

The transmission of arboviruses by mosquitoes and other arthropod vectors has considerable adverse impacts on human and animal health. This group of pathogens consists primarily of viruses in the families *Flaviviridae*, *Togaviridae* *Bunyaviridae*, and *Reoviridae* [1–4]. Arboviruses replicate in both vertebrate and arthropod hosts. In mosquitoes, arboviruses must also spread from the midgut, which is the initial site of infection following a bloodmeal to the salivary glands for transmission to another vertebrate host. The genus *Alphavirus* (family *Togaviridae*) contains several mosquito-vectored arboviruses including models like Sindbis virus (SINV) and Semliki Forest virus (SFV) [5,6] but also the re-emerging human pathogen chikungunya virus (CHIKV) [7]. The genetic structure and replication of alphaviruses, which replicate in the cytoplasm, have been analysed in detail [5,6,8,9]. All members of the genus have positive-stranded RNA genomes that are approximately 11–12 kb in size, and have 5′ caps and 3′ poly(A) tails (genetic structure of SFV shown in Fig. 1A). All alphaviruses also encode two major polyproteins. The 5′ encoded non-structural polyprotein P1234 is proteolytically cleaved into replicase proteins nsP1–4 while the 3′ encoded structural polyprotein (translated from a subgenomic mRNA, which is transcribed under control of a subgenomic promoter) is proteolytically cleaved into the structural proteins that form the capsid and envelope of the virion. The glycosylated envelope proteins play key roles in entry into cells by mediating virus binding to host cell receptor(s) and subsequent fusion to endosomes (though other entry mechanisms may be possible) while the capsid protein encapsulates the viral genome [10–12]. Infection of mosquito cell cultures has also been useful to study arbovirus replication, thus allowing increasingly detailed studies of arbovirus/vector interactions [13,14].

The innate immune system of mosquitoes plays an important role in the control of arbovirus infections, and SFV has proven to be a good models to study mosquito antiviral response mechanisms [14]. A key antiviral defence is RNAi (reviewed in [14–17]), which also influences arbovirus spread and transmission [18,19]. In addition, differential regulation of mosquito immune signalling pathways and other host genes has been described following infection by dengue virus (DENV), West Nile virus (WNV) and SINV [20–24]. JAK/STAT and Toll signalling pathways both mediate antiviral activity against DENV [23,24]. Interestingly, infection of *Anopheles gambiae* with the alphavirus o'nyong-nyong (ONNV) did not result in upregulation of the Toll and JAK/STAT pathways although other genes involved in immunity were

Author Summary

Arboviruses are transmitted to vertebrates by arthropod vectors such as mosquitoes. Infection of mosquitoes with arboviruses activates immune defence responses including the RNA interference pathway. Another component of the insect immune system is the phenoloxidase (PO) cascade, which produces melanin that accumulates at wound sites and around invading microorganisms. Some pathogen-associated pattern recognition molecules are known to activate the PO cascade, which results in the proteolytic processing of inactive prophenoloxidase (PPO) to PO. PO then catalyses the formation of compounds that ultimately form melanin. Some of these products are also known to have anti-microbial properties but whether activation of the PO cascade provides any defence against arboviruses is unclear. Using the arbovirus, Semliki Forest virus, we show that this virus activates the PO cascade. By using recombinant Semliki Forest virus expressing an inhibitor of the PO cascade, we also demonstrate that this pathway inhibits virus spread in cell culture. Moreover, inhibition of this pathway leads to higher virus genome levels and higher mortality of infected mosquitoes. In conclusion, Semliki Forest virus activates the PO cascade which exhibits antiviral activity and can be added to the list of mosquito anti-viral defence mechanisms.

upregulated with some displaying antiviral activities [25]. Innate immune signalling can also inhibit SFV replication in mosquito cells [26], while experiments in *Drosophila melanogaster* suggest that replication of SINV is inhibited by the IMD pathway [27].

Another conserved component of the insect immune system is the extracellular phenoloxidase (PO) cascade, which generates cytotoxic intermediates and the formation of melanin following wounding or infection [28–32]. Several factors have been shown to activate the PO cascade including pathogen-associated molecular pattern molecules like bacterial peptidoglycan. Other components of the cascade include multiple clip-domain serine proteases (cSPs) whose activation results in processing of the zymogen prophenoloxidase (PPO or proPO) to form active PO. PO then catalyses the conversion of mono- and di-phenolic substrates to quinones, which are converted to melanin.

A number of studies have shown that deposition of melanin provides defence against bacteria and multicellular parasites, while intermediates like 5,6-dihydroxyindole have been shown to be cytotoxic and act against pathogens [29,33,34]. Studies with the lepidopteran *Heliothis virescens* (tobacco budworm) indicate that haemolymph also contains factors with antiviral activity against *Helicoperva zea* single capsid nucleopolyhedrovirus (*Hz*SNPV) and other viruses including SINV, while bioassays with 5,6-dihydroxyindole show that it rapidly inactives *Autographa californica* multicapsid nucleopolyhedrosis virus (AcMNPV) [35–38]. Haemolymph melanisation in Lepidoptera also correlates with antiviral activity against *Microplitis demolitor* bracovirus (MdBV) [39], and *Lymantria dispar* multicapsid nucleopolyhedrovirus [40]. Whether arboviruses activate the PO cascade in mosquitoes and whether products of the PO cascade exhibit biologically relevant antiviral activity remains unclear, although interestingly RNAi knockdown of PPO I in the mosquito *Armigeres subalbatus* by a recombinant SINV expressing a dsRNA targeting PPO I resulted in reduced PO activity and higher SINV titres [41].

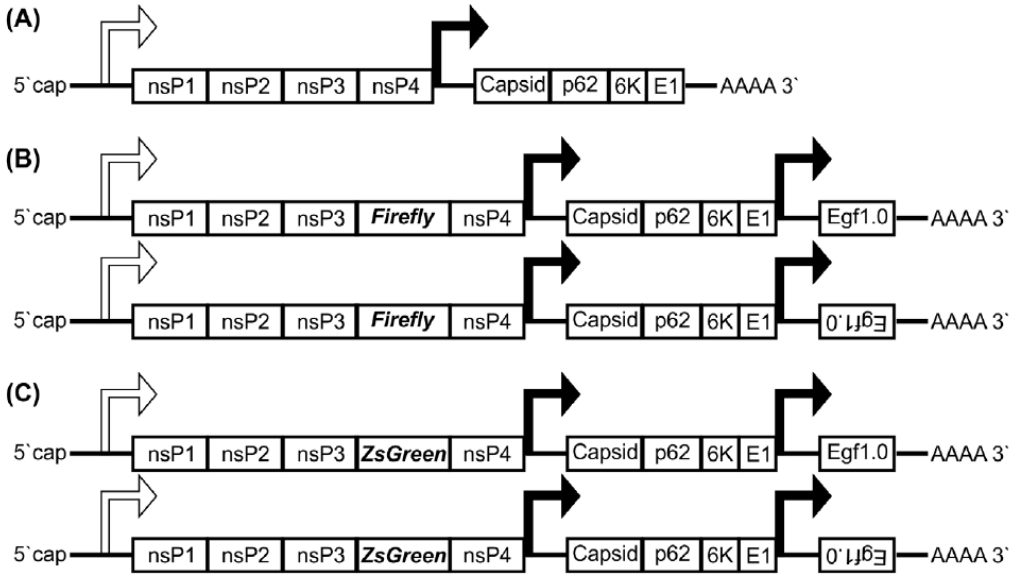

Figure 1. Viruses used in study. (**A**) SFV (prototype strain SFV4). (**B**) SFV(3H)-*FFLuc*-Egf1.0F and SFV(3H)-*FFLuc*-Egf1.0R, encoding Firefly luciferase (*FFLuc*) as part of the non-structural polyprotein (inserted between duplicated nsP2 cleavage sites at the nsP3/4 junction), and from a duplicated subgenomic promoter the melanisation inhibitor Egf1.0 in sense (F virus; top) or (as negative control) antisense orientation (R virus; bottom). (**C**) SFV(3F)-*ZsGreen*-Egf1.0F and SFV(3F)-*ZsGreen*-Egf1.0R, expressing ZsGreen inserted into the C-terminal region of nsP3, and from a duplicated subgenomic promoter the melanisation inhibitor Egf1.0 in sense (F virus; top) or (as negative control) antisense orientation (R virus; bottom).
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Previous studies show that *Aedes albopictus*-derived U4.4 cells have a functional antiviral RNAi response and immune signalling pathways [26,42]. Here we show that conditioned medium from U4.4 cells contains inducible PO activity that is activated by exposure to bacteria and purified SFV particles. Expression of the PO cascade inhibitor Egf1.0 from MdBV [39,43] by SFV decreased PO activity in U4.4 cell conditioned medium and enhanced the spread of virus through cell cultures. Infection of *Ae. aegypti* mosquitoes with SFV expressing Egf1.0 resulted in enhanced viral replication and mosquito mortality. Taken together, our results establish a role for the PO cascade in mosquito immune defence against an arbovirus.

Results

Immune challenge by bacteria and SFV increases PO activity in U4.4 cell-conditioned medium

The haemolymph of mosquitoes melanises in response to a variety of stimuli including wounding and infection [28]. Mosquitoes including *Ae. aegypti* encode multiple PPO genes, with some family members being inducibly expressed in response to microbial infection [44–47]. Haemocyte-like cell lines from *An. gambiae* also express multiple PPO genes [48], and recent studies identify cSP CLIPB9 as a candidate PAP [49].

Since the U4.4 cell line from *Ae. albopictus* is an important model for studying immune responses against arboviruses [26,42,50], we first asked whether conditioned medium from this cell line exhibited an increase in melanisation upon exposure to SFV or the bacterium *Escherichia coli* which is a well known elicitor of the PO cascade. Using a standard spectrophotometric assay for measuring melanisation activity (see Materials and Methods), our results indicated that PO activity significantly increased in U4.4 cell conditioned medium following exposure to each microbe ($p = 0.003$; *E. coli* versus control, $p = 0.004$; SFV versus control, $p = 0.021$; *E. coli* versus SFV, $p = 1.00$) (Fig. 2A). Our results also indicated that a 1 h incubation in conditioned medium significantly reduced SFV viability relative to virus incubated in unconditioned medium ($p = 0.011$) (Fig. 2B).

Because amphipathic molecules like detergents and alcohol activate insect PPOs [51], intracellular PO activity is commonly assayed for in PO producing cells like haemocytes by first fixing them in methanol and then incubating in a substrate like dopamine, which PO utilizes to produce melanin. This in turn causes the fixed cell to turn black or darken. In the case of *Ae. aegypti* and *An. gambiae*, prior studies establish that one class of haemocytes, oenocytoids, constitutively exhibit intracellular PO activity while a second class, granulocytes, inducibly exhibit intracellular PO activity following immune challenge with bacteria [52,53]. To assess whether U4.4 cells exhibit intracellular PO activity, we fixed cells in glacial methanol and then incubated them in buffer plus dopamine. Our results showed no intracellular PO activity in the majority of cells but a small fraction of cells (0.2%) darkened in manner similar to mosquito haemocytes (Fig. 2C) [52,53]. We also noted that these melanising cells display a rounded morphology and appear larger than other U4.4 cells that do not darken after fixation and incubation with substrate. We thus concluded from these assays that U4.4 cell-conditioned medium melanises following exposure to SFV or bacteria, and that a small proportion of U4.4 cells also melanise after fixation. We also concluded the increase in melanisation activity that occurs in conditioned medium correlates with a reduction in SFV viability.

Expression of Egf1.0 by SFV inhibits PO activity in U4.4 cell-conditioned medium

As previously noted, the PO cascade consists of multiple proteases that terminate with the zymogen PPO [28–32] (Fig. 3A). The number of proteolytic steps in the cascade has not been fully characterised in any insect including mosquitoes. However, it is known that infection, wounding, and other challenges trigger activation of upstream serine proteases, which result in processing of proPAPs (also referred to as pro-PPAEs or pro-PPAFs) between their clip and protease domains. Activated PAPs then process PPO by cleavage at a conserved arginine-phenylalanine (R-F) site in the N-terminal domain of the protein, which results in formation of PO (Fig. 3B). PO catalyses the hydroxylation of monophenols like tyrosine to *o*-diphenols and the oxidation of *o*-diphenols to quinones. Quinones thereafter undergo further enzymatic and non-enzymatic reactions that produce cytotoxic intermediates and ultimately melanin. Negative regulation of the PO cascade occurs through endogenous protease inhibitors like serpins, while reducing agents in haemolymph like glutathione (GSH) likely inhibit melanisation by reducing PO-generated quinones back to diphenols [54] (Fig. 3A). Several pathogenic organisms have also evolved strategies to suppress the PO cascade of hosts [28]. One of these is the virus MdBV, which produces the protein Egf1.0. Functional characterization of Egf1.0 showed that it blocks haemolymph melanisation in diverse insects including mosquitoes through two activities (Fig. 3A, B). First, it competitively inhibits activated PAPs because it contains an R-F reactive site that mimics the cleavage site for PPO [39]. Second, Egf1.0 contains another domain that prevents upstream proteases from processing pro-PAPs [43].

Given this background, we asked whether Egf1.0 could inhibit the increase in melanisation activity that occurs in U4.4 cell-conditioned medium following exposure to SFV or *E. coli*. To answer this question, we produced two sets of constructs. In the first, we cloned the *egf*1.0 gene from MdBV [39] in forward (expressing the Egf1.0 protein) and reverse (negative control not expressing Egf1.0) orientation into SFV under control of a second subgenomic promoter to produce SFV4(3H)-*FFLuc*-Egf1.0F and SFV4(3H)-*FFLuc*-Egf1.0R (Fig. 1B). These viruses also expressed Firefly luciferase (*FFLuc*), which served as an indicator for viral replication and spread through a U4.4 cell culture as previously shown for reporter gene-expressing SFV [50] (Fig. 1B). The second set of SFV constructs expressed Egf1.0 in forward or reverse orientation from a second subgenomic promoter plus ZsGreen fluorescent protein inserted into the C-terminal region of nsP3 to produce SFV4(3F)-*ZsGreen*-Egf1.0F and SFV4(3F)-*ZsGreen*-Egf1.0R, respectively (Fig. 1C).

Next, the properties of SFV-expressed Egf1.0 were analysed. We infected U4.4 cells with SFV4(3F)-*ZsGreen*-Egf1.0F and SFV4(3F)-*ZsGreen*-Egf1.0R at a multiplicity of infection (MOI) of 10. Immunoblot analysis of cell lysates confirmed that each recombinant virus actively replicated as evidenced by detection of the nsP3-ZsGreen protein (Fig. 4A). Using an anti-Egf1.0 antibody, we also detected full-length Egf1.0 [39,43] in the medium and lysates prepared from U4.4 cells infected with SFV4(3F)-*ZsGreen*-Egf1.0F but did not detect this protein in medium or lysates from uninfected cells or cells infected with SFV4(3F)-*ZsGreen*-Egf1.0R (Fig. 4A). Our Egf1.0 antibody also detected several other bands smaller than full-length Egf1.0 in samples infected with SFV4(3F)-*ZsGreen*-Egf1.0F including a 17.6 kDa protein that corresponded to the size of the C-terminal Egf1.0 fragment that prior studies showed is produced after cleavage by a PAP (Fig. 4A). Expression of Egf1.0 by SFV4(3H)-

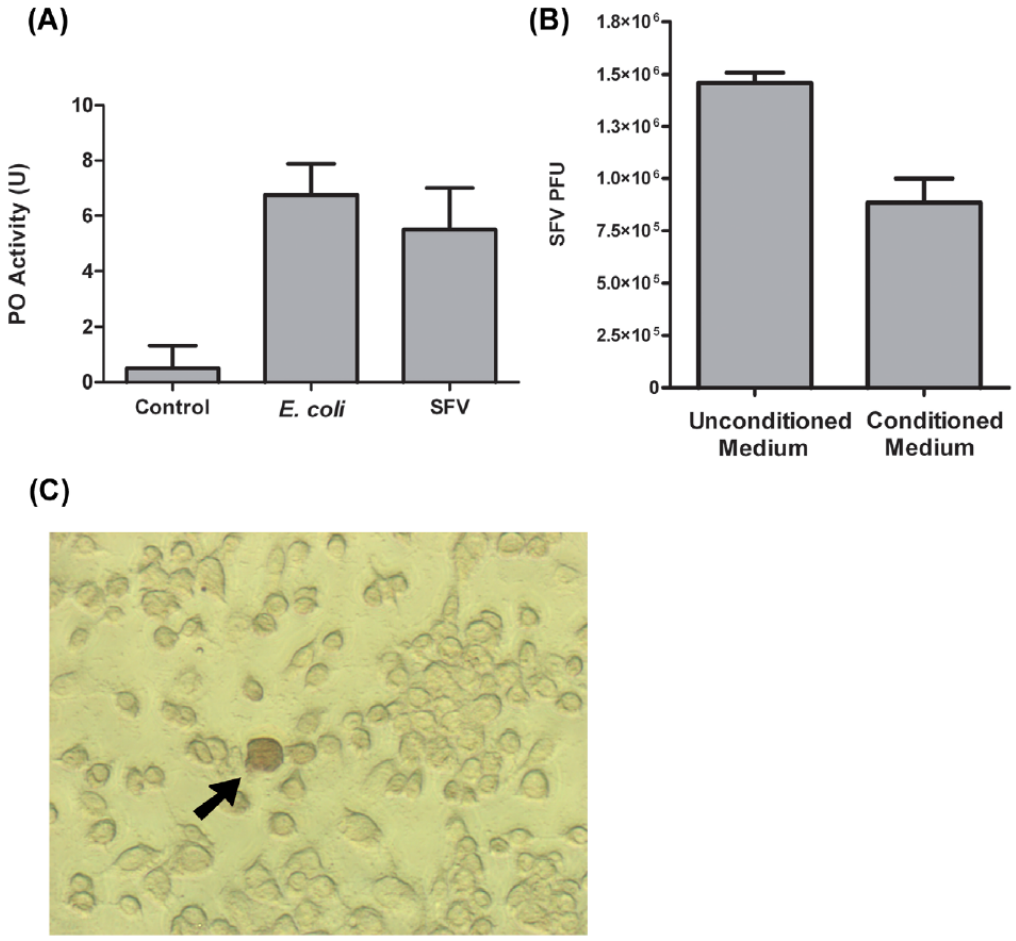
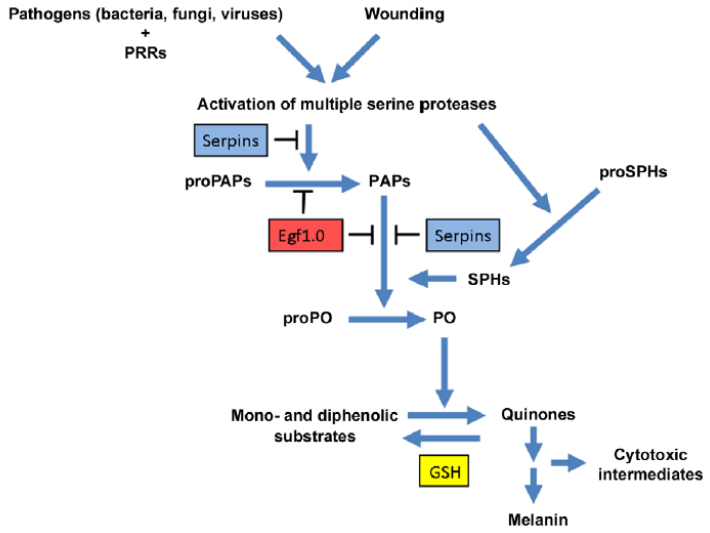


Figure 2. PO activity in U4.4 cell-conditioned medium. (**A**) PO activity in conditioned medium without immune challenge (Control) or after the addition of *E. coli* or purified SFV virions. One unit (U) of PO activity was defined as $\Delta A_{490} = 0.001$ after 30 minutes incubation (see Materials and Methods). Each bar represents the mean from 10 reactions; error bars show standard deviation. This experiment was repeated three times with similar results. (**B**) SFV virion viability after a 1 h incubation at 28°C in unconditioned culture medium or medium conditioned by U4.4 cells for 48 h. Viability was then determined by titration of SFV on BHK-21 cells. PFU: plaque forming units. Each bar represents the mean from triplicate incubations; error bars show standard deviation. This experiment was repeated three times with similar results. (**C**) Staining for intracellular PO activity in U4.4 cells. Arrow indicates a U4.4 cell that melanised after fixation and incubation with the PO substrate dopamine. Note the larger size of this cell and its rounded morphology relative to surrounding cells that have not melanised.
doi:10.1371/journal.ppat.1002977.g002

FFLuc-Egf1.0F and absence of Egf1.0 expression by SFV4(3H)-*FFLuc*-Egf1.0R were also verified by immunoblotting (not shown).

We then analyzed the functional properties of SFV-expressed Egf1.0 in conditioned medium from U4.4 cells. Melanisation assays at 48 h post-infection (p.i.) showed that conditioned medium from cells infected with SFV4(3H)-*FFLuc*-Egf1.0F exhibited very low PO activity, which was very similar and not significantly different to conditioned medium from uninfected (control) U4.4 cells ($p = 1.0$) (Fig. 4B). In contrast, medium from cells infected with SFV4(3H)-*FFLuc*-Egf1.0R exhibited PO activity levels that were significantly higher than medium from uninfected control cells ($p = 0.025$) (Fig. 4B). Conditioned medium of U4.4 cells infected with SFV4(3H)-*FFLuc*-Egf1.0F also contained significantly less (75%) PO activity than medium from cells infected with control virus SFV4(3H)-*FFLuc*-Egf1.0R ($p<0.001$) (Fig. 4B). The addition of *E. coli* to medium from SFV- infected cells had no effect on the PO activity ($p = 0.251$). As shown in Fig. 4B, the addition of *E. coli* to medium from SFV4(3H)-*FFLuc*-Egf1.0F-infected cells did not increase PO activity as would be expected if Egf1.0 was inhibiting PAP activity. Addition of *E. coli* to medium from SFV4(3H)-*FFLuc*-Egf1.0R-infected cells also did not elevate PO activity beyond the elevated level of activity that

(A)



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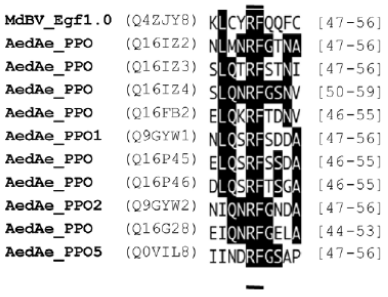

Figure 3. Activation and inhibition of the melanisation pathway. (**A**) Schematic showing the insect PO cascade and the known mode of action of Egf1.0. Infection by different pathogens and external wounding both trigger activation of the PO cascade. Proteins in the haemolymph known as humoral pattern recognition receptors (PRRs) bind to factors on the surface of different pathogens. This interaction as well as external wounding trigger the activation of multiple serine proteases. Some of these proteases have been identified in different insect species, whereas others remain unknown. Activation of these proteases leads to activation of prophenoloxidase activating proteases (proPAPs to PAPs). Some PAPs also require serine protease homologs (SPHs) for function, which are themselves activated by upstream serine proteases. PAPs cleave proPO (also called PPO) to PO, which oxidizes mono- and diphenolic substrates to quinones that undergo further reactions to form melanin. A number of these intermediate products are cytotoxic including some that have also been shown to inactivate viruses (see Text). Serine protease inhibitors called serpins have been identified from different species of insects that inhibit PAPs or other proteases in the pathway. In the absence of wounding or infection, the reducing agent glutathione (GSH) also exists in haemolymph at concentrations that can inhibit melanisation by recycling quinones to diphenols. The inhibitor Egf1.0 from MdBV inhibits both the processing of proPAPs and PAPs that have already been activated. (**B**) Alignment of the reactive site loop of Egf1.0 to the predicted cleavage sites for the PPOs encoded by *Ae. aegypti* (indicated as AedAePPO). Note the identical P1–P1′ residues R-F (underlined) of Egf1.0 and PPO family members. Black highlighting indicates identical residues. UniProt database identifiers in parentheses to the left of the alignment.
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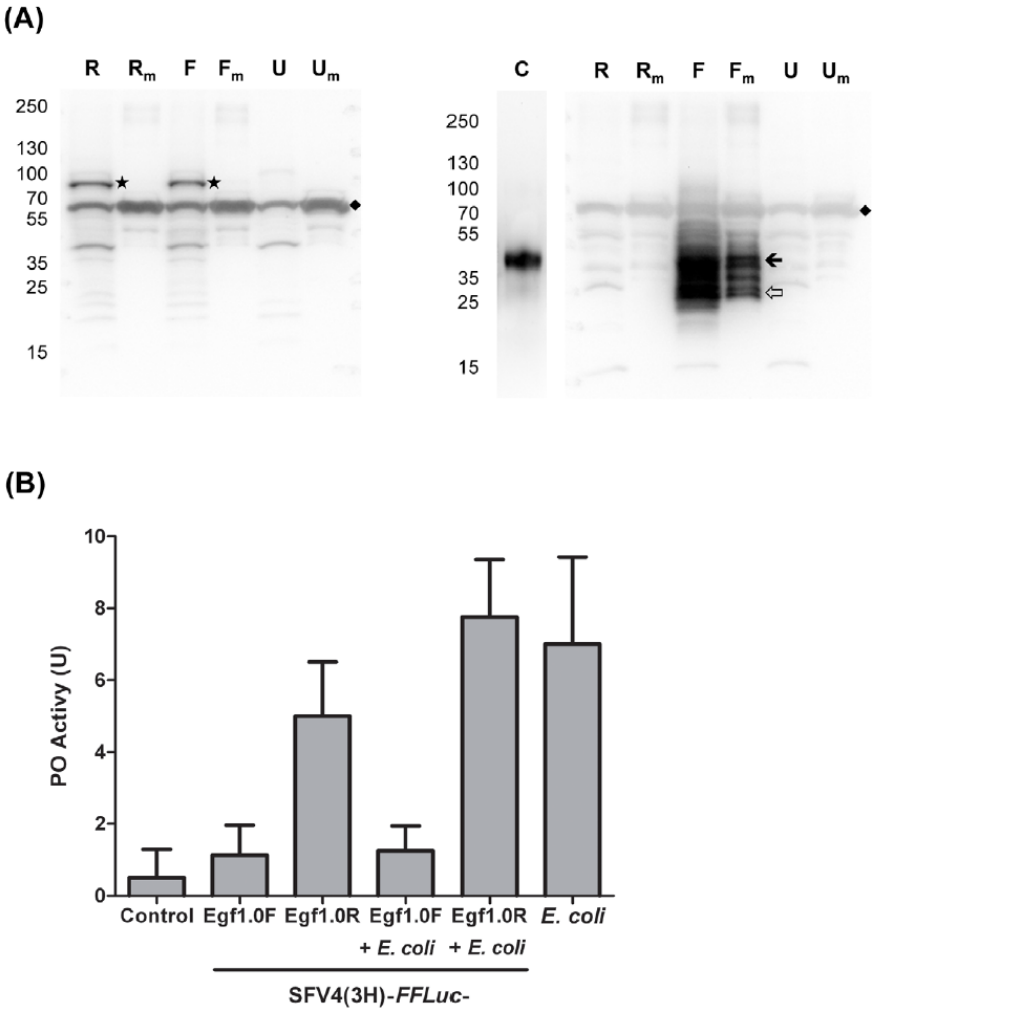

Figure 4. Recombinant SFV expresses Egf1.0 and inhibits PO activity in U4.4 cell-conditioned medium. (**A**) Immunoblots showing Egf1.0 expression and secretion from mosquito cells. U4.4 cells were infected with SFV4(3F)-*ZsGreen*-Egf1.0F or SFV4(3F)-*ZsGreen*-Egf1.0R at an MOI of 10 followed by preparation of cell lysate and medium samples at 48 h p.i. as indicated in the Materials and Methods. The left blot was probed with an anti-SFV nsP3 antibody with individual lanes labeled as follows: U4.4 cells infected with SFV4(3F)-*ZsGreen*-Egf1.0R (R = cell lysate, Rm = conditioned medium), U4.4 cells infected with SFV4(3F)-*ZsGreen*-Egf1.0F (F = cell lysate, Fm = conditioned medium), or uninfected cells (U = cell lysate, Um = conditioned medium). Black star identifies the nsP3-ZsGreen protein, only detected in lysates from SFV-infected cells. Black diamond indicates bovine serum albumin (non-specifically detected because of high abundance). The right blot shows the same samples probed with an anti-Egf1.0 antibody. A control lane (C) was added to this blot (purified, recombinant Egf1.0). Note that Egf1.0 is only detected in the control lane, and F and Fm lanes. Black arrow indicates uncut Egf1.0; open arrow identifies a band corresponding to the predicted C-terminal domain of Egf1.0 after PAP cleavage. Molecular mass markers indicated to the left. (**B**) PO activity in conditioned medium from uninfected U4.4 cells (Control), cells infected with SFV4(3H)-*FFLuc*-Egf1.0F (Egf1.0F), SFV4(3H)-*FFLuc*-Egf1.0R (Egf1.0R), cells infected with SFV4(3H)-*FFLuc*-Egf1.0F with *E. coli* added to the medium (Egf1.0F+*E. coli*), cells infected with SFV4(3H)-*FFLuc*-Egf1.0R with *E. coli* added to the medium (Egf1.0R+*E. coli*), or medium from uninfected cells with *E. coli* added (*E. coli*). PO activity was measured as outlined in Fig. 2A; 1 ml of conditioned medium was taken at 48 h p.i. from 2.6×10^5 U4.4 cells infected at an MOI of 10, or uninfected (Control). Each bar represents the mean from 10 reactions; error bars show standard deviation. This experiment was repeated three times with similar results.
doi:10.1371/journal.ppat.1002977.g004

already existed. Taken together, these results showed that SFV4(3H)-*FFLuc*-Egf1.0F produced Egf1.0 in U4.4 cells, which is secreted into the medium. Given prior evidence that Egf1.0 specifically inhibits the PO cascade by disabling PAP function, these data also strongly suggested that U4.4 cell-conditioned medium contains a functional PO cascade, which is activated by SFV or gram-negative bacteria, and which is inhibited by SFV-produced Egf1.0.

The inhibitor Egf1.0 enhances SFV spread through U4.4 cell culture

We next asked whether inhibition of PO activity by Egf1.0 could enhance virus spread during an infection. We first used our SFV4(3H)-*FFLuc*-Egf1.0F or SFV4(3H)-*FFLuc*-Egf1.0R constructs which allowed us to monitor viral replication and spread through a U4.4 cell culture by measuring *FFluc* activity at 24 h and 48 h p.i., similar to previously described experiments [50]. Infections were carried out at either a high multiplicity of infection (MOI 10), where most U4.4 cells were infected and little or no further spread of virus could occur, or a low MOI (0.005) where only a small fraction of cells were initially infected and SFV could thereafter disseminate through the medium to infect other cells. Overall GLM revealed differences in *FFLuc* activity as a function of MOI (10 or 0.005), construct (SFV4(3H)-*FFLuc*-Egf1.0F or SFV4(3H)-*FFLuc*-Egf1.0R) and sample time (24 h or 48 h p.i.) (Fig. 5 A&B, $p = 0.012$). As a result the data from the high and low MOI treatments were examined separately.

At an MOI of 10, cells infected with SFV4(3H)-*FFLuc*-Egf1.0F or SFV4(3H)-*FFLuc*-Egf1.0R exhibited similar levels of *FFluc* activity at 24 h or 48 h p.i. ($p = 0.74$) (Fig. 5A). This outcome was fully consistent with most cells being infected and containing actively replicating SFV, while also indicating that Egf1.0 had no effect on intracellular replication activity. As expected, rates of replication also dropped to low levels for both recombinant viruses at 48 h p.i. ($p<0.001$) as they each entered the persistent phase of infection [26] (Fig. 5A). In contrast, we observed a very different outcome when cells were infected at a low MOI where *FFluc* activity differed between cells infected with SFV4(3H)-*FFLuc*-Egf1.0F or SFV4(3H)-*FFLuc*-Egf1.0R. At 24 h p.i, there was no difference in *FFLuc* activity between cells infected with SFV4(3H)-*FFLuc*-Egf1.0F and SFV4(3H)-*FFLuc*-Egf1.0R ($p = 0.37$), but at 48 h p.i. SFV4(3H)-*FFLuc*-Egf1.0F showed significantly higher spread and replication rates than SFV4(3H)-*FFLuc*-Egf1.0R ($p = 0.004$) (Fig. 5A). We reasoned that this difference was also most likely linked to the time required for Egf1.0 to be expressed and secreted, and infectious SFV to be produced.

Repeating these experiments using SFV4(3F)-*ZsGreen*-Egf1.0F and SFV4(3F)-*ZsGreen*-Egf1.0R allowed us to visualize virus spread from one cell to another through the green fluorescing foci that form from ZsGreen presence in viral replication complexes (ZsGreen is inserted into the C-terminal region of nsP3; Fig. 1C). At a high MOI of 10, most U4.4 cells contained green foci at 48 h when infected with SFV4(3F)-*ZsGreen*-Egf1.0F or SFV4(3F)-*ZsGreen*-Egf1.0R (Fig. 5B). At a low MOI of 0.005, however, more cells exhibited green foci at 48 h p.i. when infected with SFV4(3F)-*ZsGreen*-Egf1.0F than SFV4(3F)-*ZsGreen*-Egf1.0R (Fig. 5B).

Overall, these data strongly suggested that activation of the PO cascade by SFV reduced virus spread, whereas Egf1.0 enhances virus spread by inhibiting the PO cascade. However, these results did not provide any insight into the identity of the effector molecules produced by the PO cascade that reduce SFV viability and spread. To assess whether the anti-SFV effects of PO were due to the formation of reactive intermediates or other products formed by PO, we infected U4.4 cells with a low MOI of SFV4(3H)-*FFLuc*-Egf1.0R (MOI 0.005) and added GSH (0.5 mM), which as noted above likely inhibits melanisation by reducing quinones (see Fig. 3A) [54]. Our results showed that GSH significantly increased the spread of SFV4-*FFLuc*-Egf1.0R relative to medium without added GSH ($p<0.001$). As expected though, the addition of GSH did not change the rate of spread of SFV4(3H)-*FFLuc*-Egf1.0F ($p = 0.139$) (Fig. 6A).

Although vertebrates lack a PO cascade, we also tested whether expression of Egf1.0 conferred a replicative advantage to SFV in BHK-21 cells. There was no significant difference in the spread of SFV4(3H)-*FFLuc*-Egf1.0F and SFV4(3H)-*FFLuc*-Egf1.0R ($p = 0.64$) following low MOI infection (0.005), indicating that Egf1.0 had no effect on dissemination of SFV in this mammalian cell line (Fig. 6B).

PO activity protects mosquitoes following SFV infection

Immunologically important antiviral pathways in mosquitoes such as RNAi have been previously implicated in promoting mosquito survival after arbovirus infection. Indeed, inhibition of the RNAi pathway through alphavirus-expressed RNAi inhibitors results in rapid death of virus-infected mosquitoes [55,56]. To test whether the PO cascade provides an effective antiviral defence in mosquitoes, we extended our experiments to *Ae. aegypti*, a mosquito species that is generally relevant as an arbovirus vector, and which has also been shown to transmit SFV in the laboratory [57–59]. Prior studies also implicate *Ae. aegypti* alongside *Ae. africanus* as a natural vector of SFV [60]. *Ae. aegypti* were fed bloodmeals containing SFV4(3H)-*FFLuc*-Egf1.0F, SFV4(3H)-*FFLuc*-Egf1.0R, or no virus (mock-infection). We then monitored mosquito survival (cohorts of 22–25 mosquitoes) following infection in three independent experiments to determine survival rates (Fig. 7A). Since no significant differences were detected within treatments in the three experiments ($p>0.05$), the samples were pooled for further analysis. Overall, mosquito survival differed significantly among treatments (Kaplan Meier $\chi^2 = 25.37$; $p<0.001$). Post Hoc multiple comparison tests revealed no significant difference in survival rates between the mock-infected control and mosquitoes infected with SFV4(3H)-*FFLuc*-Egf1.0R ($p = 0.98$). In contrast, mosquitoes infected with SFV4(3H)-*FFLuc*-Egf1.0F exhibited higher mortality than mock-infected mosquitoes ($p<0.001$) or mosquitoes infected with SFV4(3H)-*FFLuc*-Egf1.0R ($p<0.001$). In conclusion, inhibition of the PO cascade decreased survival following infection of mosquitoes with SFV.

To assess whether the reduced survival of SFV4(3H)-*FFLuc*-Egf1.0F-infected mosquitoes was associated with enhanced viral replication, mosquitoes (cohorts of 10) were fed bloodmeals containing SFV4(3H)-*FFLuc*-Egf1.0F or SFV4(3H)-*FFLuc*-Egf1.0R. Total RNA was then extracted at 3 days post-bloodmeal followed by qPCR analysis to determine SFV genome copy number per individual. This time point was chosen because it just precedes quantifiable differences in mosquito survival, thus avoiding mortality-induced bias. Our results showed that viral genome copy numbers were higher in mosquitoes fed SFV4(3H)-*FFLuc*-Egf1.0F than in mosquitoes fed SFV4(3H)-*FFLuc*-Egf1.0R (Mann-Whitney test, $p = 0.04$) (Fig. 7B). Interestingly, infection rates were also higher when mosquitoes were infected with SFV4(3H)-*FFLuc*-Egf1.0F than SFV4(3H)-*FFLuc*-Egf1.0R (Fig. 7B). This suggests that Egf1.0-mediated inhibition of the PO cascade is also potentially important in establishment of an infection. Higher infection rates have been previously observed with alphaviruses expressing RNAi inhibitors or following silencing of antiviral RNAi genes during mosquito infection [55,61].

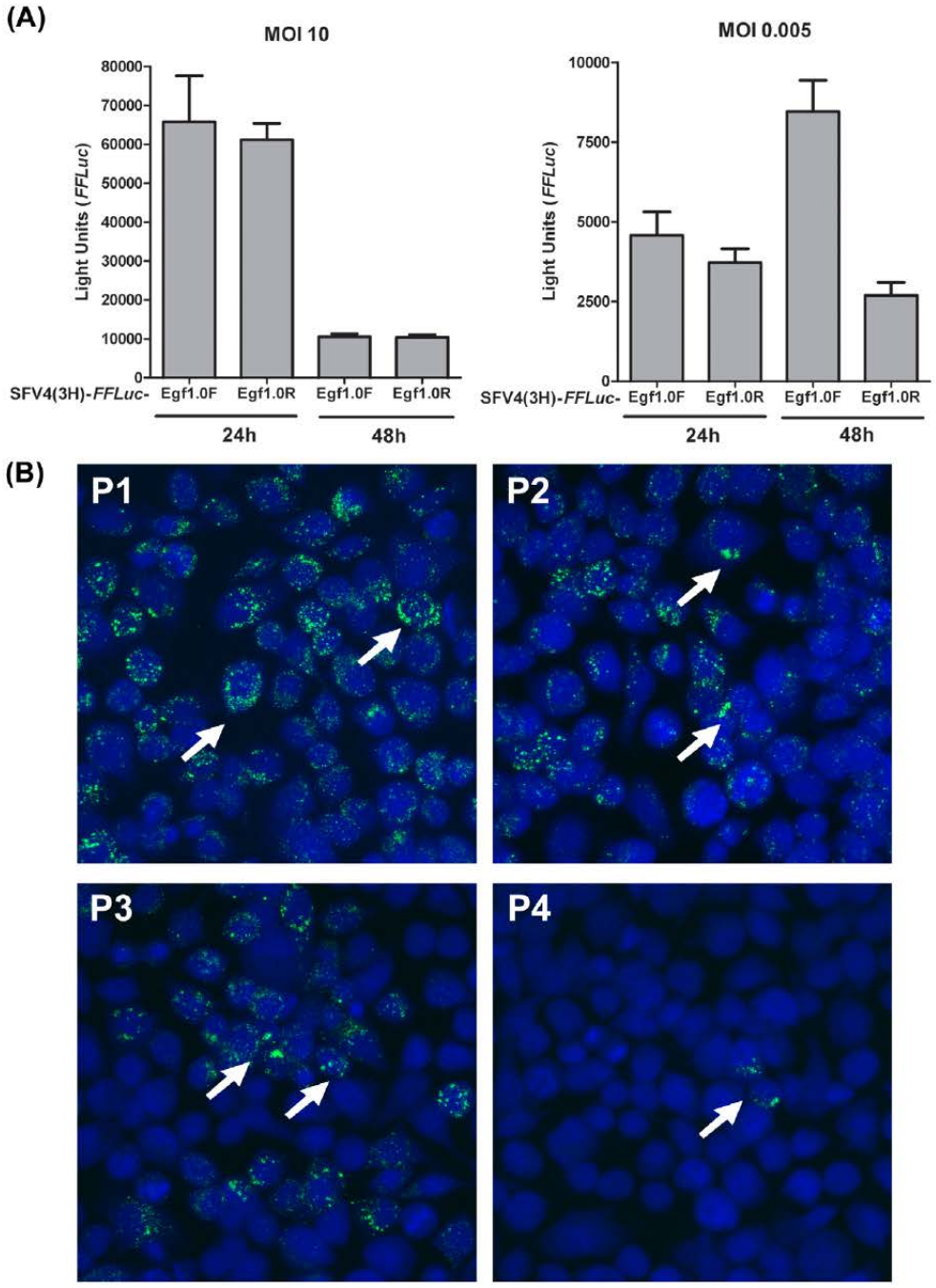

Figure 5. Inhibition of PO activity enhances the spread of SFV in U4.4 cells. (**A**) U4.4 cells were infected with SFV4(3H)-*FFLuc*-Egf1.0F (Egf1.0F) or SFV4(3H)-*FFLuc*-Egf1.0R (Egf1.0R) at an MOI of 10 (left graph) or 0.005 (right graph). *FFLuc* activity (expressed in lights units) was determined at 24 or 48 h p.i. Each bar represents the mean from triplicate cultures; error bars show standard deviation. This experiment was repeated

three times with similar results. (**B**) Epifluorescent micrographs of U4.4 cells 48 h p.i. with SFV4(3F)-*ZsGreen*-Egf1.0F at an MOI of 10 (P1), SFV4(3F)-*ZsGreen*-Egf1.0F at an MOI of 0.005 (P3), SFV4(3F)-*ZsGreen*-Egf1.0R at an MOI of 10 (P2), or SFV4(3F)-*ZsGreen*-Egf1.0R at an MOI of 0.005 (P4). Arrows point to ZsGreen-positive replication foci in the cytoplasm of selected U4.4 cells. Cell nuclei are counterstained with marker TOPRO3. Note at low MOI infection the increased number of replication foci in cells infected with SFV4(3F)-*ZsGreen*-Egf1.0F (P3) relative to cells infected with SFV4(3F)-*ZsGreen*-Egf1.0R (P4). This experiment was repeated three times with similar results.
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Discussion

Comparative genome analysis of different mosquito species reveals a noticeable expansion of PPO genes relative to other insects. For example, *An. gambiae* encodes nine PPOs while *Ae. aegypti* encodes ten. Expansion in the numbers of clip-domain serine proteases and serpins has also occurred [47]. The recent sequencing of the *Culex quinquefasciatus* genome reveals nine PPOs and thirty-two serpins, compared to originally twenty-three serpins in *Ae. aegypti* though recent studies and Vectorbase increase this number to twenty-six [45–47,62]. Compared to other insects including *An. gambiae*, relatively little is known about regulation of the PO cascade in mosquitoes although recent studies in *Ae. aegypti* identify some of the processes involved [62]. Interestingly the cSP family also contains proteins with non-catalytic protease domain, so-called clip domain serine protease homologs (cSPHs), and both cSPs and cSPHs (as co-factors) are involved in melanisation reactions.

In *Ae. aegypti* and *An. gambiae*, cSPs and cSPHs are divided into five subfamilies called CLIP A, B, C, D and E [47]. Mainly CLIP B subfamily proteases are known (or suggested) to activate PPOs. Melanisation in *Ae. aegypti* was found to be regulated by protease inhibitor Serpins-1, -2 and -3 which regulate different cSPs [62]. In that study, two separate pathways leading to PPO cleavage were described; a first pathway linking Serpin-1 to (CLIP B subfamily members) Immune melanisation protease (IMP)-1 and IMP-2, and a second pathway linking Serpin-2 to Tissue melanisation protease (TMP) and IMP-1. Depletion of Serpin-2 leads to tissue melanisation and appears to be involved in activation of the Toll pathway, while depletion of Serpin-1 leads to immune responses against the parasite *Plasmodium gallinaceum* [62]. Other regulators of melanisation in *Ae. aegypti* such as CLSP2 (a modular protein consisting of C-type lectin and elastase-like domains) have been described [63]. Transcription of at least some PPO genes in *Ae. aegypti* is also regulated by the Toll pathway [44], thus linking different branches of the immune response.

Based on the antiviral activities of insect haemolymph [35,36], we hypothesized that immune reactions induced by PO extend to arboviral infection of mosquitoes. Our experiments collectively indicate that U4.4 cell-conditioned medium contains a functional PO cascade. Our detection of a small proportion of U4.4 cells that melanise after fixation and incubation with dopamine further suggest these cells are likely source of the PO activity detected in conditioned medium. Notably, these cells morphologically resemble oenocytoids, which also comprise less than 1% of the circulating haemocyte population in mosquitoes like *Ae. aegypti* and *An. gambiae* [52,53] as well as many other insects, yet are also the primary source of PO in plasma [51]. Ongoing analysis of the U4.4 cell transcriptome indicates that PPO orthologs are expressed although at this time it remains unclear whether expression is restricted to the large, rounded cells that stain after incubation with dopamine or is more global. Regardless of these uncertainties, our results strongly indicate that medium conditioned by U4.4 cells contains a functional PO cascade that is activated by exposure to SFV or *E. coli*, and is inhibited by Egf1.0. Prior studies in Lepidoptera show that MdBV also activates the PO cascade [39] while bacterial cell wall components like peptidoglycan are well known activators of the PO cascade in a

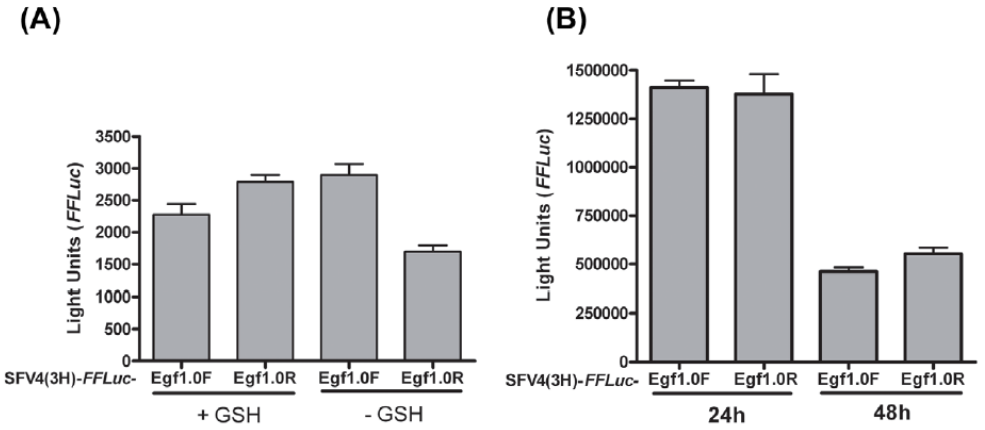

Figure 6. Spread of SFV in mosquito and vertebrate cells. (**A**) The addition of glutathione (GSH) to medium enhances the spread of SFV. U4.4 cells were infected with SFV4(3H)-*FFLuc*-Egf1.0F (Egf1.0F) or SFV4(3H)-*FFLuc*-Egf1.0R (Egf1.0R) at an MOI of 0.005 followed by determination of *FFLuc* activity at 48 h p.i. + GSH: 0.5 mM GSH; − GSH: negative control. Each bar represents the mean from triplicate cultures; error bars show standard deviation. This experiment was repeated three times with similar results. (**B**) Egf1.0 has no effect on SFV spread in BHK-21 cells. Cells were infected with SFV4(3H)-*FFLuc*-Egf1.0F (Egf1.0F) or SFV4(3H)-*FFLuc*-Egf1.0R (Egf1.0R) at an MOI of 0.005 followed by determination of *FFLuc* activity at 24 h and 48 h p.i. Each bar represents the mean from triplicate cultures; error bars show standard deviation. This experiment was repeated three times with similar results.
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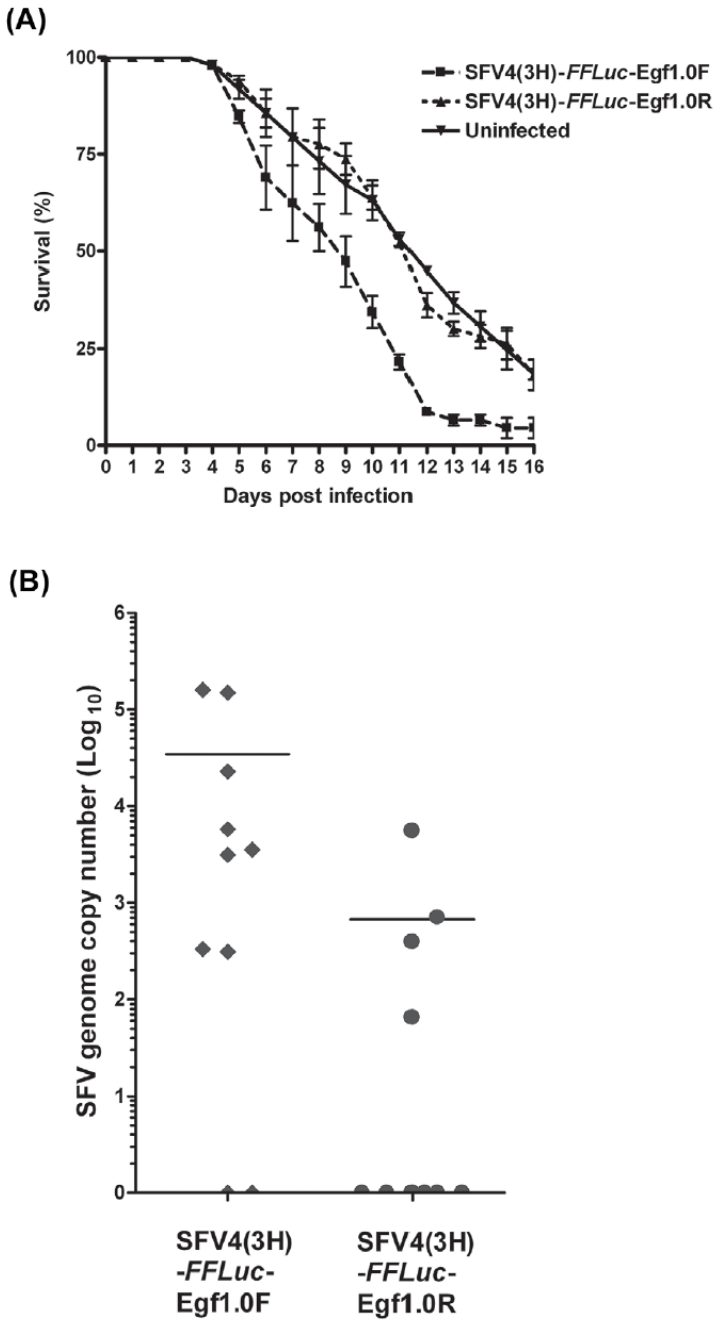

Figure 7. Expression of Egf1.0 increases mortality of *Ae. aegypti* and replication of SFV *in vivo*. (**A**) *Ae. aegypti* were fed blood containing SFV4(3H)-*FFLuc*-Egf1.0F or SFV4(3H)-*FFLuc*-Egf1.0R. Uninfected blood meals served as a control. Mosquito mortality was then monitored daily post-bloodmeal. Combined survival data from three independent experiments (cohorts of 22–25 infected mosquitoes per virus or control mosquitoes in each experiment) are shown. Error bars show standard deviation. (**B**) SFV genome copy number as determined by real time qPCR. Total RNA was

extracted 3 days post-bloodmeal from mosquitoes infected with SFV4(3H)-*FFLuc*-Egf1.0F or SFV4(3H)-*FFLuc*-Egf1.0R. Viral genome RNA levels from 10 mosquitoes for each virus are shown. Values at 0 represent uninfected mosquitoes. Horizontal bar indicates average genome copy number from infected mosquitoes. This experiment was repeated three times with similar results.
doi:10.1371/journal.ppat.1002977.g007

diversity of insects [28–32]. We think it likely that activation of the PO cascade in U4.4 cell-conditioned medium by *E. coli* similarly involves binding of bacterial cell wall components by currently unknown humoral pattern recognition receptors. In contrast, it remains unclear what features of SFV induce a similar increase in PO activity. One possibility is that glycoproteins of the viral envelope function as pathogen-associated molecular patterns. The lectin pathway of vertebrate complement is known to be activated by pattern recognition receptors such as mannose–binding lectin that binds mannose-containing glycoproteins [64]. Several lectins have also been described as candidate pattern recognition receptors in insects [65].

While additional studies will be needed to identify how SFV is being recognised in U4.4 cell conditioned medium, our results collectively indicate that activation of the PO cascade and the associated increase in melanisation that occurs reduces the spread of SFV among the U4.4 cell population. Reduced survival of *Ae. aegypti* combined with enhanced virus replication when mosquitoes are infected by SFV expressing Egf1.0 also suggests the PO cascade is important in limiting arbovirus spread in mosquitoes. Interestingly, gene expression data obtained following ONNV infection of *An. gambiae* indirectly suggest that ONNV infection may have led to activation of melanisation pathways in the early stages of infection [25], which highlights the importance of this study.

On the other hand, the effects of PO cascade inhibition on mosquito survival are most apparent at later stages post-bloodmeal compared to experiments with alphaviruses expressing RNAi inhibitors [55,56]. This suggests that inhibition of the PO cascade takes more time than disruption of RNAi or that this response is less powerful than RNAi in defence against arboviruses. However these experiments show that viral expression of an inhibitor is a viable strategy for inhibiting insect immune responses. Expression from the subgenomic promoter of recombinant SFV results in high levels of Egf1.0 and strong inhibitory activity, which may be difficult to achieve by just silencing a target gene through RNAi. Thus, an important goal for future studies will be to assess how inhibition of the PO cascade affects the spread of SFV in different tissues of mosquitoes as well as how the PO cascade may interact with other immune defence responses including the RNAi pathway.

Previous experiments where PPO I was silenced in *Ar. subalbatus* by expression of PPO I dsRNA using recombinant SINV showed increased titres of SINV [41]. Our results take this observation further by showing that activation of the PO cascade reduces SFV viability *in vitro* and that Egf1.0-mediated inhibition enhances virus replication and spread both *in vitro* and *in vivo*. However it is not entirely clear what products generated by the PO cascade are responsible for the antiviral activity against SFV we observe. Given the antiviral properties of 5,6-dihydroxyindole against AcMNPV [38], and the ability of GSH to inhibit anti-SFV activity in conditioned U4.4 cell culture medium suggests that the reactive intermediates generated by PO are antiviral. However, it is also possible the PO cascade might reduce arbovirus spread from the initial site of infection through the production of melanin and/or activation of other signaling pathways like Toll or IMD that also have roles in antiviral defence. To distinguish between these possibilities will require studies that directly assess the effects of 5,6-dihydroxyindole, melanin, or other compounds on the integrity of SFV virions [38]. Any damage to structural proteins could result in failure to bind receptors and/or enter cells. Questions also remain over the tissue specificity of PO activity. Our *in vitro* and *in vivo* data overall suggest products of the PO cascade may be antiviral because they reduce the viability of virions in the haemocoel. However other research describes melanisation reactions in the extracellular space between *An. gambiae* midgut cells following *Plasmodium berghei* infection [66]. Thus inhibition of PO activity by Egf1.0 could enhance SFV replication and spread in or around midgut tissues. Finally, our study does not directly address the question of whether wild-type SFV can potentially inhibit or evade the PO response. Given though that SFV spread is enhanced by expression of a powerful inhibitor like Egf1.0, we suspect the ability of wild-type SFV to inhibit or evade host-associated PO defence response is likely weak.

Alphaviruses are not inhibited by the Toll pathway in insects [26,27], but links between the PO cascade and Toll signalling in *Ae. aegypti* could, as noted above, play a role in antiviral defence. Infection of *Ae. aegypti* with DENV-2 results in differential regulation of serpins although it is not possible yet to speculate whether these have a role in controlling PPO activation [24]. It does however suggest that protease-mediated antiviral defences extend to other arbovirus families. Intriguingly, it has been shown that infection of insects with strains of endosymbyotic *Wolbachia* bacteria, which can inhibit arbovirus infection by yet unknown mechanisms [67], may upregulate melanisation or genes involved in melanisation [68,69]. Thus, our findings also may explain in part the antiviral properties mediated by *Wolbachia* infection. Future work will determine whether these findings also extend to viruses from other arbovirus families.

Materials and Methods

Ethics statement

Under UK Home Office legislation insects such as mosquitoes are not considered animals. No animals were used in the course of these experiments. Defibrinated sheep blood was obtained from TCS Biosciences (Buckingham, United Kingdom).

Cells, viruses and infection

Ae. albopictus-derived U4.4 mosquito cells were grown at 28°C in L-15 medium with 10% fetal calf serum and 10% tryptose phosphate broth. BHK-21 cells were grown in Glasgow minimum essential medium (GMEM) with 10% newborn calf serum and 10% tryptose phosphate broth at 37°C in a 5% CO_2 atmosphere. Amplification of SFV (strain SFV4) and recombinant clones derived from SFV4 in BHK-21 cells (grown as described above), together with titration of plaque forming units (PFU) in BHK-21 cells have been previously described [50]. SFV and derived clones were purified from supernatant as described and resuspended in TNE (Tris-NaCl-EDTA) buffer [70]. Viruses were diluted in PBSA (PBS with 0.75% bovine serum albumin) and added to U4.4 cells at room temperature for 1 h followed by washing twice to remove any unbound particles; cells were grown at 28°C following infection. Details of reporter viruses (Fig. 1) can be obtained from the authors. The pCMV-SFV4 backbone for production of SFV4 has been previously described [71]. A second subgenomic promoter was placed behind the SFV4 structural open reading

frame for construction of viruses with duplicated subgenomic promoters [72]. This second subgenomic promoter is of the T37/17 type (consisting of a sequence 37 nucleotides upstream and 17 nucleotides downstream of the original transcription start-site of the SFV subgenomic mRNA). The ZsGreen marker was inserted into the C-terminal region of nsP3 via a *Xho*I site naturally occuring in the genomic sequence (leading to expression of nsP3 containing ZsGreen), while Firefly luciferase (*FFLuc*) was inserted between duplicated nsP2 cleavage sites at the nsP3/4 junction as a cleavable reporter, using strategies previously shown [73]. The full *egf1.0* coding sequence (including signal peptide) derived from MdBV was placed under control of the second subgenomic promoter in sense or antisense (as negative control) orientation.

Detection of ZsGreen expression

Cells on glass slides were fixed in 10% formaldehyde (Fisher Chemicals) for 45 min and washed in PBS three times. Cells were treated with TO-PRO 3 (Invitrogen) (1:1000) in dH2O for 10 min and washed with PBS three times. Slides were mounted using Vectashield mounting medium (Vector Laboratories). Cells and fluorescence were then visualised by confocal microscopy.

Detection of Egf1.0 by immunoblotting

At 48 h p.i., U4.4 cells infected with SFV (MOI of 10) or control uninfected cells were lysed in Laemmli buffer. Conditioned cell culture medium was concentrated on Millipore Centricon-Plus 70 Centrifugal Filter Units prior to addition of Laemmli buffer. Recombinant Egf1.0 produced as previously described [39] served as a positive control. Samples were run on a 4–20% Tris-Gycine PAGEr precast gels (Lonza), and blotted onto Immobilon-P PVDF membranes (Millipore). SFV infection was detected using a rabbit anti-nsP3 antibody (1:20000), while Egf1.0 was detected using a rabbit anti-Egf1.0 antibody (1:35000) [39,74]. Primary antibodies were detected using a horseradish peroxidase (HRP)-conjugated goat-anti rabbit secondary antibody (Jackson ImmunoResearch) (1:45000), followed by visualisation using the ECL Advance Western Blotting Kit (Amersham) and a GeneGnome bioimaging system (Syngene).

Mosquito rearing and infection

Aedes aegypti (Liverpool red eye strain optimised for filarial growth) were kindly provided by R. M. Maizels and Y. Harcus (Institute of Immunology and Infection Research, University of Edinburgh). Mosquitoes were kept at 27°C, in 85% humidity and with a 16 h light: 8 h dark photoperiod. Larvae were fed on a standard yeast diet, while adults were fed on 10% fructose continuously. Female adults were 4 to 5 days old when allowed to feed on defibrinated sheep blood (TCS Biosciences) containing 5×10^7 PFU of virus per ml of blood supplemented with 4 mM ATP. Mosquitoes were starved for 24 h before feeding and the bloodmeal (at 37°C) provided by a Hemotek membrane feeder (Discovery Workshops, Accrington, UK) for 2 h. Mosquitoes that fed were removed and maintained at standard conditions with fructose.

Melanisation assays and determination of PO activity

Conditioned cell culture medium from *Ae. albopictus*-derived U4.4 mosquito cells was harvested 48 h post-cell seeding (4×10^6 cells in a 75 cm^2 flask) and centrifuged at 2000 rpm for 5 min in order to eliminate residual cells. Approximately 5 µl of a pelleted *E. coli* JM109 culture (New England Biolabs) or 3.5×10^7 PFU of SFV were added to 1 ml of cell culture medium and incubated for 10 min at room temperature. The mixture was then centrifuged at 3000 rpm for 10 min at 4°C in order to remove debris. Following this, PO activity assays were carried out in 96-well plates with 100 ul of 50 mM Sodium Phosphate buffer (pH 6.5) containing 2 mM dopamine added to 20 µl of cell culture medium [75]. PO activity was monitored by measuring absorbance at 490 nm using a plate reader (Dynatech MR5000) over a period of 30 min. It should be noted that this approach predominantly detects dopachrome and/or dopaminechrome rather than melanin itself. One unit of PO activity was defined as $\Delta A_{490} = 0.001$ after 30 minutes, similar to previously described [39,76,77]. For each experimental condition, PO activities from 10 reactions were determined. Intracellular PO activity was assessed by first fixing U4.4 cells in glacial methanol. After rinsing in PBS, fixed cells were then incubated for 1 h in phosphate buffer plus 2 mM dopamine.

Determination of luciferase activities

Following cell lysis in Passive Lysis Buffer (Promega), luciferase activities were determined by using a Dual Luciferase assay kit (Promega) on a GloMax 20/20 luminometer.

Real time quantitative PCR analysis (qPCR)

SFV4 genome copy number was quantified as previously described [26]. Briefly, total RNA was isolated from single *Ae. aegypti* using Trizol (Invitrogen). RNA quality and quantity were assessed with a NanoDrop 1000 spectrophotometer (Fisher Scientific). A total of 0.5 µg of total RNA per mosquito was reverse transcribed using Superscript III kit (Invitrogen) and oligo-dT primer, and reactions were analysed in triplicate. The reaction mix contained 0.8 µM of each primer, FastStart SYBR Green Master x1 (Roche), and 2 µl of template. Tubes were heated to 94°C for 5 min, and then cycled through 94°C for 20 sec, 62°C for 20 sec, and 72°C for 20 sec for 40 cycles on a RotorGene 3000 instrument (Corbett Research). Sequences of the primers were as indicated: 5′ -GCAAGAGGCAAACGAACAGA-3′ (SFV-nsP3-for) and 5′ –GGGAAAAGATGAGCAAACCA-3′ (SFV-nsP3-rev). The number of SFV genome copies was calculated using a standard curve generated with the plasmid pSFV1.

Statistical analysis

Data with 2 groups were analysed using either t-test or Mann Whitney tests, depending on the structure of the data. Data with more than 2 groups was analysed using General Linear Models (GLM). All GLMs were initially performed including all fixed effects and their interactions. Any post hoc tests were adjusted for multiple comparisons using the Bonferroni correction. Survival analysis was performed on cohorts of 22–25 mosquitoes. Differences between survivorship curves were tested using Kaplan-Meier estimator and the log-rank test. Where appropriate, multiple comparisons were performed and the Bonferroni correction was applied. All analyses were conducted using SAS v9.1.3 (SAS Institute Inc., Cary, NC, USA). Diagnostics were performed and plots of residuals were examined, confirming the goodness-of-fit of all models. Prior to analysis, it was specified that results with $p<0.05$ would be reported as exhibiting formal statistical significance.

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Author Contributions

Conceived and designed the experiments: JRA MRS JKF AK. Performed the experiments: JRA SR MV MHB MCF RF ES GB. Analyzed the data: JRA MECT MHB MRS JKF AK. Contributed reagents/materials/analysis tools: MRS AM. Wrote the paper: AK MRS JRA JKF.

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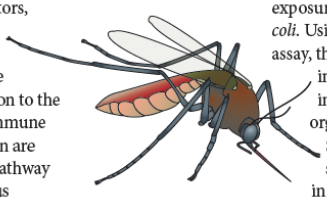
HOST RESPONSE

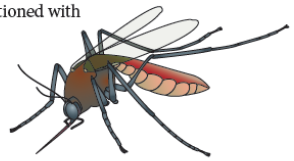
PO-faced response to SFV

Arboviruses such as Semliki Forest virus (SFV) replicate in both vertebrate hosts and mosquito vectors, where they must spread from the initial site of infection in the mosquito midgut to the salivary glands for transmission to the next host. Mosquito innate immune defences to arbovirus infection are known to include the RNAi pathway as well as the JAK/STAT (janus kinase–signal transducer and activator of transcription) and Toll signalling pathways. Writing in *PLoS Pathogens*, Rodriguez-Andres *et al.* now identify the phenoloxidase (PO) cascade as an additional weapon in the antiviral armoury of the mosquito.

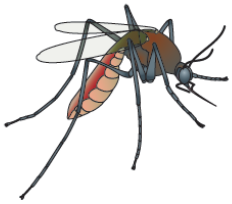


The PO cascade is an extracellular pathway in insects that is triggered by certain pathogen-associated molecular patterns and consists of a cascade of clip-domain serine proteases that lead to processing of the zymogen pro-PO to produce active PO. When activated, PO converts monophenolic and diphenolic compounds into quinones, which undergo further reactions to produce cytotoxic intermediates and, ultimately, melanin, which has been shown to encapsulate certain pathogens. To determine whether arboviruses activate the PO cascade, the authors began by investigating whether growth medium conditioned with the U4.4 cell line from *Aedes albopictus* became melanized following exposure to either SFV or *Escherichia coli*. Using a spectrophotometric assay, the authors observed an increase in PO activity following exposure to each microorganism. They also found that SFV viability decreased substantially following incubation in U4.4-conditioned medium, suggesting that the PO cascade had antiviral activity.

> “U4.4 cells infected with Egf1.0-expressing virus at a low multiplicity of infection … exhibited a substantial increase in the spread and rate of infection”



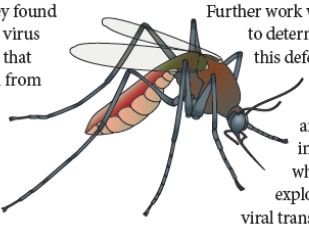
The *Microplitis demolitor* bracovirus protein Egf1.0 is known to block haemolymph melanization in a range of arthropods by competitively inhibiting proteases that activate pro-PO. The authors cloned the *egf1.0* gene into SFV together with a reporter gene that allowed viral replication to be monitored. They found that the recombinant virus expressed Egf1.0 and that conditioned medium from U4.4 cells infected with this virus exhibited almost no PO activity. Importantly, U4.4 cells infected with Egf1.0-expressing virus at a low multiplicity of infection (0.005) exhibited a substantial increase in the spread and rate of infection compared to cells infected with a virus that did not express Egf1.0. Furthermore, addition of glutathione (which inhibits melanization by reducing quinones) to the medium increased the spread of viruses lacking Egf1.0 to levels approaching those of viruses that expressed the inhibitor.



Finally, to test whether the PO cascade is an effective antiviral defence in mosquitoes, the authors fed a blood meal containing either a control or an Egf1.0-expressing virus to *Aedes aegypti*, which had previously been shown to transmit SFV in the laboratory. They found that expression of Egf1.0 led to increased viral replication, which correlated with higher mortality for the mosquito vector.

These studies identify the PO cascade and melanization as important components of the mosquito vector's response to infection with SFV. Further work will be needed to determine whether this defence pathway is common to other arbovirus–vector interactions and whether it can be exploited to limit viral transmission.

Andrew Jermy



ORIGINAL RESEARCH PAPER Rodriguez-Andres, J. *et al.* Phenoloxidase activity acts as a mosquito innate immune response against infection with Semliki Forest virus. *PLoS Pathog.* **8**, e1002977 (2012)