

Imperial College London

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**Functional and phenotypic
characterization of the Stearoyl CoA
desaturase gene of *Anopheles coluzzii***

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of the requirements for the degree of
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Declaration of Originality

I certify that the thesis and research to which it refers to are solely my own work and the works and ideas from other people which are being used in the thesis are fully acknowledged in accordance with standard referencing practices.

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Abstract

Malaria is an infectious disease caused by *Plasmodium* parasites that are transmitted by the bite of female *Anopheles* mosquitoes. Successful acquisition and transmission of malaria parasites requires a female mosquito obtaining a blood meal from human hosts. The blood meal, which is rich in protein, is required for egg development. Most of the ingested protein is converted to lipid and stored in the fat body where vitellogenesis takes place. In this process, saturated fatty acids are converted to unsaturated fatty acids by the stearoyl-CoA desaturase (SCD1). Unsaturated fatty acids are also essential for maintaining cell membrane fluidity and other housekeeping functions. The main aim of this thesis was to functionally and phenotypically characterize the function of SCD1 during blood meal metabolism in the African mosquito vector *Anopheles coluzzii*.

RNA interference (RNAi) silencing of the *SCD1* gene and administration of a small molecule inhibitor of SCD1 had a significant impact on the survival of female mosquitoes following a blood meal. *SCD1* knockdown (KD) caused a 100% mortality within 48 h after a human blood meal, while addition of the SCD1 small molecule inhibitor sterculic acid (SA) in the blood meal caused a 50% mortality within 72 h of blood meal. Microscopic analysis showed that *SCD1* KD mosquitoes failed to develop eggs in response to the blood meal, while their thorax was filled with blood at 24 h post blood meal. These findings were highly consistent with electron microscopy data that showed increased plasma membrane rigidity and depletion of lipid droplets in the midgut epithelial cells. Transcriptional profiling using *A. coluzzii* oligonucleotide DNA microarrays showed that genes involved in protein, lipid and carbohydrate metabolism, as well as a large number of immunity genes were the most affected in blood-fed *SCD1* KD versus control mosquitoes. Metabolomics profiling highlighted the biochemical framework by which the *SCD1* KD phenotype is manifested after a blood meal, revealing increased amounts of saturated fatty acids and TCA cycle (and other interlinked pathway) intermediates in *SCD1* KD and SA-treated mosquitoes.

The data reported in this thesis reveal that silencing of *SCD1* in female *A. coluzzii* mosquitoes leads to a metabolic syndrome primarily associated with the increase of saturated fatty acids and TCA cycle intermediates, which affects important biological functions leading to premature mosquito death. The accumulation of saturated fatty acids is also the likely cause of a potent immune response observed in the absence of infection,

which resembles an auto-inflammatory reaction. These data provide important leads for the development of novel interventions aiming to block transmission of mosquito-borne diseases.

To My Family

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Abbreviations

20-hydroxyecdysone	20E
3-hydroxy-3-methylglutaryl-CoA lyase	HMG-CoA lyase
Acetyl CoA carboxylase	ACC
Adenylosuccinate lyase	ADL
Adenosine triphosphate	ATP
<i>Aedes aegypti</i>	<i>Ae. aegypti</i>
<i>Aedes albopictus</i>	<i>Ae. albopictus</i>
Alanine aminotransferase	ALAT
Aminopeptidase N1	APN1
<i>Anopheles coluzzii</i>	<i>A. coluzzii</i>
<i>Anopheles gambiae</i>	<i>A. Gambiae</i>
Antimicrobial peptides	AMPs
Apolipoprotein	Apo
Argininosuccinate lyase	ASL
Argininosuccinate synthase	ASS
Argininosuccinate synthase	ASS
Carnitine-palmitoyl-transferase	CPT
Cecropin 1	CEC1
Chikungunya	CHK
chitin-binding protein	CBP
Clip-domain serine proteases	CLIP
Cluster of Differentiation80	CD80
Collagen IV	CLG IV
Cytochrome c oxidase polypeptide 7A	COX7A
Damage/danger-associated molecular patterns	DAMPs
Dengue	DEN
Deoxyribonucleic acid	DNA
Diacylglycerol	DAG
Dihydroxyacetone phosphate	DHAP
Dihydroxyacetone phosphate	DHAP
Double stranded RNA	DsRNA
<i>Drosophila melanogaster</i>	<i>D. melanogaster</i>
Endoplasmic reticulum	ER
Eukaryotic initiation factor 4E	eIF4E
Fibrinogen related proteins	FREP
Free fatty acid	FFA
Galactokinase	GALK
Gambicin1	GAM1
Glutathione-S-transferase A1	GSTA1

Glutathione-S-transferase A2	GSTA2
Glutathione-S-transferase D7	GSTD7
Glyceraldehyde-3- phosphate	GAP
Glycogen synthase-kinase	GSK3- β
Guanosine triphosphate	ADP
Guanosine diphosphate	GDP
Guanosine triphosphate	GTP
Hour	H
Indoor residual spraying (IRS)	IRS
Insulin-like peptides	ILPs
Japanese encephalitis	JE
Juvenile hormone	JH
Juvenile hormone binding protein	JHBP
Knock down	KD
Lactate dehydrogenase	LDH
Lateral cell membrane	LCM
Lipid droplet	LD
Lipid-binding MD2-like protein	ML
Lipophorin	Lp
long-lasting insecticidal nets (LLINs)	LLINs
lysosomal membrane proteins	LAMP
Mono unsaturated fatty acid	MUFA
odorant binding protein	OBP
Ornithene decarboxylase	ODC
Ornithine decarboxylase	ODC
Pathogen associated molecular patterns	PAMP
Peritrophic matrix	PM
Phenylalanine hydroxylase	PAH
Phosphatidylinositol 3-kinase	PI3K
Phosphatidylinositol4-kinase type 2	PI4K
Phosphatidylinositol4-phosphate	PI4P
Phosphoenol pyruvate carboxykinase	PEPCK
Phosphoenolpyruvate carboxykinase	PEPCK
Phospholipid scramblase	PLS1
Phosphotyrosine protein phosphatase	ptyr_pPase
Post blood meal	PBM
Post drug feeding	PDF
Pro-phenoloxidases	PPO
PUFA	Poly unsaturated fatty acid
Pyruvate carboxylase	PC
<i>Plasmodium falciparum</i>	<i>P. falciparum</i>
RNA Binding Motif Protein 15	RBM15
RNA interference	RNAi
Saturated fatty acid	SFA
Stearoyl-CoA desaturase 1	SCD1
Sterculic acid	SA
Sterculic oil	SO

Sterol regulatory element binding protein1	SREBP1
Succinate CoA ligase	SUCL
Succinate CoA synthetase	SUCS
TCA cycle	TCA cycle
The target of rapamycin	TOR
Thioester-containing protein	TEP
Toll- Like Receptors	TLR
Toll-like receptor 4	TLR4
Translation initiation factor binding protein	4E-BP
Transmission Electron Microscopy	TEM
Trehalose transporter 1	TRET1
Triglycerol	TAG
Tripartite Motif Containing 37	TRIM37
UDP glucuronosyltransferase	UGT
Vector-borne infectious diseases	VBDs
Vitellogenin	Vg
West Nile fever	WNF
Yellow Fever	YEF
Yolk protein precursor	YPP

Chapter 1

General Introduction

Summary

Vector-borne infectious diseases (VBDs) are illnesses affecting hosts (humans or other warm blooded animals) and are transmitted mainly by the bite of the vectors which are infected with pathogens or parasites. Among the 22 documented VBDs, malaria is the oldest and the most life- threatening mosquito borne disease. Successful acquisition and transmission of malaria parasites requires a female mosquito obtaining a blood meal from human hosts. The ingested blood meal is directly taken in the mosquito midgut. In the posterior midgut of a female mosquito, the ingested blood is digested into amino acids, chiefly by the activity of blood meal-induced trypsin-like proteinase enzymes. Mosquitoes use amino acids for protein synthesis and also for fatty acid biosynthesis from reduced carbons derived from amino acid. The fat body is the central storage depot for excess nutrients in the form of triglycerides and glycogen. Lipophorin transports lipid to the ovaries during egg development and 90% of total lipid of the egg is extra-ovarian. Fatty acids, the building blocks of TAG and other fats are either saturated (SFA) or unsaturated (UFA) in this process of lipid biosynthesis; saturated fatty acids are converted to unsaturated fatty acids by the stearoyl-CoA desaturase (SCD1). SCD is considered a pivotal enzyme in the body, since the ratio of stearic acid (saturated fatty acid) and oleic acid (unsaturated fatty acid) plays an important role in the maintenance of cell membrane fluidity and cell-cell interactions.

Vector-borne diseases focusing malaria:

Vector-borne diseases (VBDs) are viral, bacterial and parasitic diseases transmitted by, commonly, blood sucking arthropods and affecting humans or other warm blooded animals (Confalonieri 2007). The best-known vectors of VBDs include mosquitoes, ticks, sandflies, triatomine bugs and blackflies (Lemon 2008). They transmit diseases that affect urban, peri-urban and rural settings, especially among poor communities, and play a vital role in the spread of poverty and under-development of economies (World Health Organization 2014). VBDs constitute an increasing threat to global public health and economy due to their sensitivity to the complex dynamic of environmental and social factors that affect their spread. Ecological changes such as climate change, deforestation, building of dams, major irrigation schemes and urbanization, and social elements such as malnutrition, population displacement, poor housing, weak immune systems and lack of resources are associated with the intensity of VBD transmission (Negev et al. 2015).

Vectors, global distribution and transmission of VBDs

Major VBDs:

Among the 22 documented VBDs, the World Health Organization (WHO) has included malaria, yellow fever, West Nile fever, Japanese encephalitis, Chikungunya and other 'neglected tropical diseases' such as Dengue, visceral leishmaniasis, lymphatic filariasis and human American & African trypanosomiasis. These diseases pose a major public health concern in tropical and subtropical areas for current and future human wellbeing (World Health Organization 2014). About 8 of these 22 VBDs are transmitted to human hosts by mosquitoes (Table 1.1) and contribute a major portion of the burden of morbidity and mortality attributed to VBDs throughout the world (Hill et al. 2005). Malaria, dengue, yellow fever, chikungunya fever and lymphatic filariasis are transmitted between humans through bites of infected mosquitoes, while some VBDs like Japanese encephalitis (JE), West Nile virus fever (WNVF) can be also transmitted to humans from animals through infected mosquito bites (Fig.1. 1).

Table 1.1: Vectors and human pathogens of VBDs

Main vector	Disease	Pathogen
<i>Anopheles</i> mosquitoes	Malaria	Protozoan parasites: <i>P. falciparum</i> , <i>P. vivax</i> , <i>P. malariae</i> , <i>P. ovale</i> , <i>P. knowlesi</i>
<i>Aedes</i> mosquitoes	Dengue	Flavivirus: DENV1, DENV2, DENV3, DENV4
	Chikungunya	Alphavirus: CHIKV
	Yellow fever	Flavivirus: YEFV
	Rift valley fever	Phlebovirus: RVFV
<i>Culex</i> mosquitoes	Japanese encephalitis	Flavivirus: JEV
	West Nile fever	Flavivirus: WNV
	Lymphatic filariasis	Nematode roundworms: <i>Wuchereria bancrofti</i> , <i>Brugia malayi</i> and <i>B. timori</i>
<i>Phlebotomus</i> and <i>Lutzomyia</i> sandflies	Leishmaniasis	Kinetoplastid protozoa: <i>Leishmania donovani</i> , <i>L. infantum</i> , <i>L. major</i> , <i>L. mexicana</i>
Genera <i>Hyalomma</i> ticks	Crimean-Congo haemorrhagic fever	Nairovirus
<i>Ixodes scapularis</i>, <i>I. ricinus</i> and <i>I. persulcatus</i>	Tick-borne encephalitis	Flavivirus
<i>Ixodes ricinus</i>, <i>Ixodes persulcatus</i>	Lyme disease	Bacterium: <i>Borrelia burgdorferi</i>
<i>Amblyomma maculatum</i>	Rickettsial diseases	Bacteria: <i>Rickettsia</i> spp.
<i>Triatomine</i> bugs	Chagas disease (American trypanosomiasis)	Protozoan parasite: <i>Trypanosoma cruzi</i>
Tsetse flies	Sleeping sickness (African trypanosomiasis)	Protozoan hemoflagellates: <i>Trypanosoma brucei</i>
Fleas	Plague	Bacterium: <i>Yersinia pestis</i>
Black flies	Onchocerciasis (river blindness)	Parasitic worm: <i>Onchocerca volvulus</i>
Aquatic snails	Schistosomiasis	Trematode: <i>Schistosoma</i> spp.

SOURCE: WHO 2015

Vectors and their feeding behaviour:

Malaria is the deadliest mosquito-borne disease worldwide. It is caused by protozoan parasites of the genus *Plasmodium* and transmitted exclusively through the bites of hematophagous *Anopheles* mosquitoes (Fig. 1.1A). There are about 500 different *Anopheles* species documented so far, 60 of which are recognised as vectors of diseases. In a global context, 20 of these species are important. The most common vectors in Africa are *A. gambiae*, *A. coluzzii*, *A. arabiensis*, *A. funestus* and *A. nili* (Zofou et al. 2014). *A. dirus* complex, *A. aconitus*, *A. annularis*, *A. minimus* complex, *A. sinensis* complex, *A. stephensi*, *A. barbirostris* and *A. subpictus* complex are the most notable vectors in the Asia-Pacific region and *A. darling*, *A. albimanus* and *A. pseudopunctipennis* are primary vectors in South America and Central America (Sinka et al. 2011). The long life span and strong human biting preference of African *Anopheles* mosquitoes compared to other malaria vectors has been a main cause of the disease prevalence in Africa, accounting for 90% of global malaria deaths (Zofou et al. 2014).

The anthropophilic and cosmopolitan mosquitoes- *Aedes aegypti* and *Ae. albopictus* are the principal vectors of fast emerging and pandemic-prone viral diseases such as dengue and chikungunya in many parts of the world (Thiberville et al. 2013). The sharing of principal vectors and the overlap between chikungunya-affected and dengue-endemic areas increase the opportunity for co-infections (Chahar et al. 2009).

Other *Aedes* mosquitoes transmitting urban yellow fever include *Ae. africanus*, *Ae. simpsoni*, *Ae. furcifer* and *Ae. luteocephalus*, as well as tree-hole breeder mosquitoes such as *Masoni africana*. The yellow-fever virus (YEFV) can also exhibit vertical transmission from one mosquito generation to another via mosquito eggs (Diallo et al. 2000). Apart from *Aedes* mosquitoes, several other insects such as phlebotomine flies, horse mosquitoes (Brazil), common ticks (*Amblyomma variegatum*) in West Africa, and other parasitic arthropods have been documented as being vectors for YEFV.

Chief vectors of Rift Valley Fever in East and South Africa are flood water-borne *Aedes*, such as *Ae. cumminsii*, *Ae. circumluteolus*, and *Ae. mcintoshi*, transmitting the virus from mosquito to vertebrates or between mosquitoes through vertical transmission (Fontenille et al. 1998). All of the abovementioned *Aedes* mosquitoes can actively bite hosts throughout the day, especially in the early morning and late afternoon hours and are known to exhibit both outdoor and indoor (only *Ae. aegypti*) feeding habits.

Lymphatic filariasis that is also transmitted by mosquitoes is an acute chronic illness in tropical and sub-tropical regions. It is caused by three species of parasitic worms, which can live for an average of 6-8 years in a host body and produce millions of microfilariae (immature larvae). The urban and semi-urban *Culex* mosquitoes (*Culex quinquefasciatus*), the rural *Anopheles* mosquitoes and *Aedes* mosquitoes are the major vectors of the Bancrofti filariasis, while mosquitoes of the genus *Mansonia* are principal vectors of Brugian filariasis in south-east Asia. *Bancrofti filariasis* vectors bite both in day-time (certain *Aedes* species) and night-time (*Culex quinquefasciatus*). *Brugian filariasis* vectors can be also diurnal (*Anopheles*) and nocturnal (*Mansonia*). After blood feeding, female vectors rest either indoors (endophily) or outdoors (exophily) until their eggs are fully developed (World Health Organization 2014) .

Japanese encephalitis (JE), the leading flavivirus encephalitis in Asia, is principally transmitted to humans from vertebrate hosts (pigs, horses and/ or water birds) via the bite of an infected *Culex* mosquito (*Culex tritaeniorhynchus* and *Culex vishnui*). Humans are 'dead-end' or incidental hosts, as viraemia is generally too low to infect mosquitoes. Pigs and horses, on the other hand, are amplifier hosts and ardeid wading birds are primarily maintenance hosts (Fig.1.1B). As a result, this disease is predominant in the rural and peri-urban areas where humans live in closer proximity to the abovementioned warm - blooded vertebrates (Jansen et al. 2008). These, chiefly nocturnal, exophagic mosquitoes bite hosts from dusk till dawn and significantly increase their human biting proportion during the hot season due to the availability of humans sleeping outdoors (Reuben et al. 1992).

West Nile fever is caused by the neurotropic West Nile virus (WNV) and is transmitted in an avian cycle by ornithophilic mosquitoes, mainly of the genus *Culex*. Similarly to JE, mammals (humans or horses) are considered dead-end hosts, while birds are the main reservoir of the virus (Fig.1B). To date, over 65 mosquito species have been documented to be infected by WNV globally, including the well-accepted, primary global transmission vector *C. tarsalis*, some other *Culex* species (*C. quinquefasciatus*, *C. stigmatosoma*, *C. thriambus*, *C. pipiens*, *C. nigripalpus*), and *C. salinarius* and *Aedes* mosquitoes. Infection in male mosquitoes with WNV supports the vertical transmission ability of the virus. "Host switching" behaviour from birds to humans and mammals of *C. pipiens* and *C. tarsalis* during late summer and early autumn, has also been reported in the United States (Colpitts et al. 2012).

Global distribution and burden of VBDs:

VBDs cause a significant fraction of the global infectious disease burden, which is generally greatest in developing countries (Fig. 1.2). Each year there are more than 1 billion cases of VBD infected humans, which accounts for more than 17% of all infectious diseases, while 1

million people die, which is over 10% of global deaths, and leading to an annual 50 million disability-adjusted life years (DALYs) (Hotez et al. 2006)..

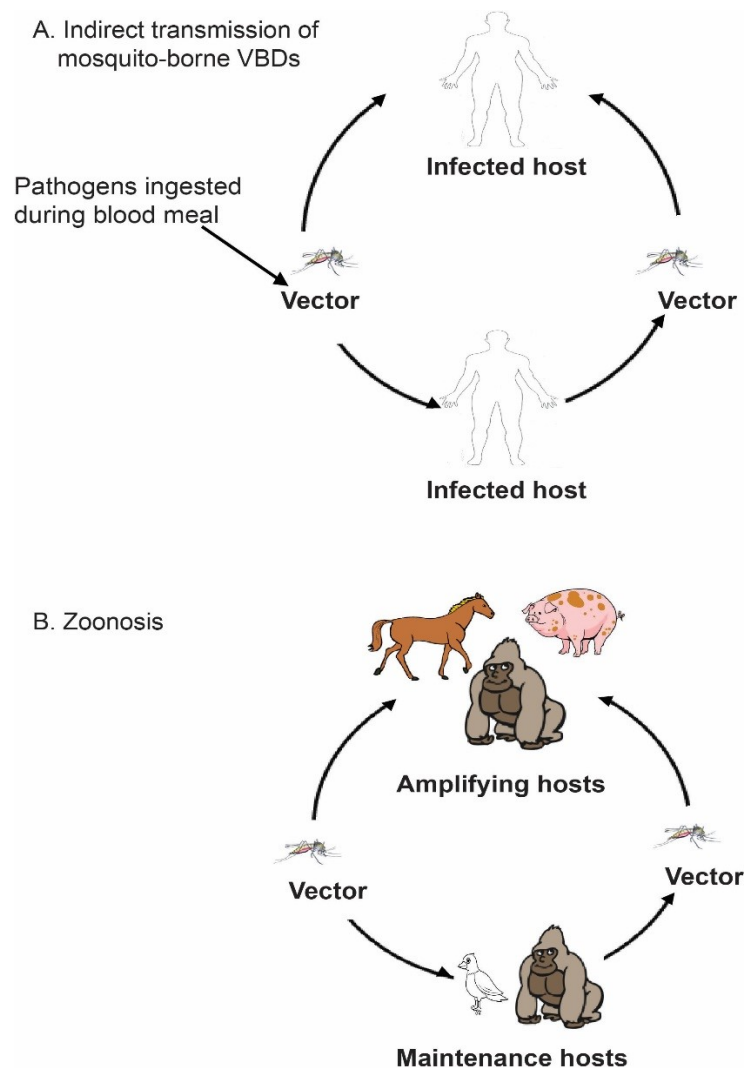


Fig. 1.1 Transmission of major mosquito borne VBDs

(A) Malaria parasites, DENV, CHKV, YEV and parasitic worms of lymphatic filariasis are spread through a human-to-mosquito-to-human cycle of transmission. Uninfected mosquitoes ingest pathogens through blood feeding on infected person and release the pathogen to a new, healthy person during the second blood meal. (B) RVFV, JE and WNV are maintain and amplify through animal-to-mosquito-to-animal cycle. Humans become infected through an infected mosquito bite but cannot pass the virus to any other organism.

Table 1.2: Estimated annual global burden of major vector-borne diseases in 2013

Disease	Estimated cases per year (millions)	Number of countries at risk
Mosquito-borne		
Malaria	198 (5,84,000) ^a	97 (3.4) ^b
Dengue*	50-100	128
Chikungunya	1 (1,000) ^a	60
Yellow fever	0.2 (30,000) ^a	44 (0.9) ^b
Japanese encephalitis	0.068 (10,000) ^a	24 (3.0) ^b
Lymphatic filariasis	120	58 (1.23) ^b
West Nile virus fever	Data not available	
Sand fly- borne infection		
Leishmaniasis	1.3 (20,000-30,000) ^a	13

The total estimated cases/year represent 17% of the global infectious and parasitic disease burden.

*Estimated cases of dengue fever for the Americas, South-east Asia and western Pacific region (other regional data is not available); ^anumber within bracket is showing the estimated number of death cases; ^bnumber within bracket is showing the estimated number of people, in a billion, who are at risk.

Source: WHO, 2015

More than half of the world's population are estimated to be at risk of VBDs (Table 1. 2). Dengue, in particular, is an emerging serious public health threat and over 40% of the global human population (approximately 2.5 billion) are at risk of contracting it. Children (mainly under 5 years of age) are significantly more vulnerable to VBDs (one child dies every minute from malaria alone) compared to adults due to their less-developed immune systems, immature organs, which can be damaged easily during infection, and a lack of awareness for health risks. The most devastating scenarios of malaria mortality are for the Democratic Republic of Congo and Nigeria, where malaria is linked to 4 in every 10 deaths recorded (World Health Organization 2014).

Most VBDs are prevalent in tropical and sub-tropical regions of the world (Fig. 1.2). Even though malaria, dengue and visceral leishmaniasis have been the major public health concern in South-East Asia for decades, JE has recently been considered as a substantial public health problem with endemic potential across Asia, especially in India, Nepal and Sri Lanka. Approximately 11% of total global VBD burden has been documented in the Eastern Mediterranean Region, where over 570 million people live. Dengue is endemic in this region.

Among all other VBDs, malaria burden is highly concentrated in sub-Saharan African countries, where nine out of ten globally reported malaria deaths occur.

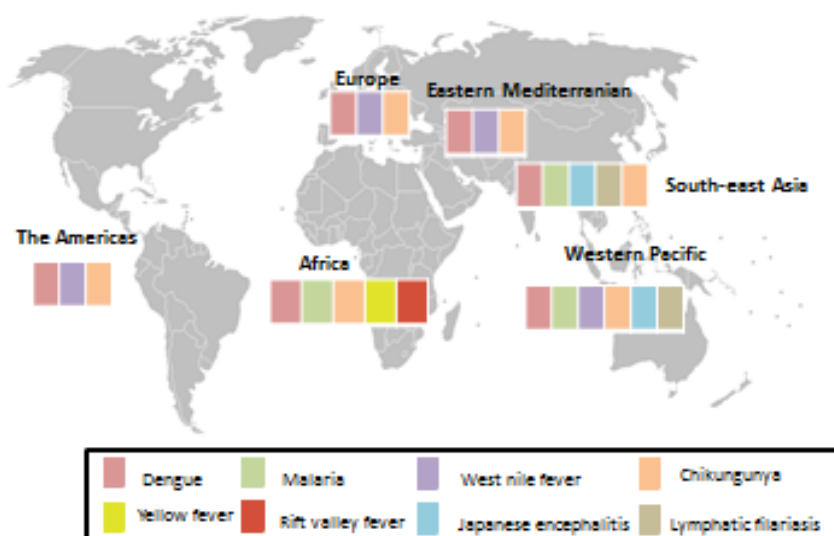


Fig.1. 2 Global distribution of major mosquito-borne VBDs

VBDs are significantly concentrated in developing tropical and subtropical countries. Malaria cases are concentrated mainly in Africa. Dengue cases are reported from almost every continent. Adapted from WHO, world malaria report, 2012.

Malaria

Malaria is one of the oldest infectious diseases caused by apicomplexan protozoan parasites of the genus *Plasmodium* and transmitted most commonly by *Anopheles* mosquitoes. Among the five species of *Plasmodium* parasites causing malaria in humans (Table 1.1), *P. falciparum* is the most fatal, contributing to 90% of malaria deaths in Africa and 50 % of malaria deaths in South-East Asia and Latin America. The disease was first clinically described by the Greek physician Hippocrates sometime in 460 BC – 370 BC. The causative agent of the disease was first observed in the red blood cells of malaria patients by French doctor Charles Louis Alphonse in 1880. Sir Ronald Ross and Battista Grassi independently

demonstrated that *Anopheles* mosquitoes are transmitting malaria to humans in the beginning of the 19th century (Bruce-Chwatt, Gilles et al 1993).

Global Malaria distribution and burden in the 21st century:

Human malaria is thought to have evolved 50,000 years ago in tropical Africa and may be a contemporary of modern human evolution. Eventually, the disease became endemic in this region due to the rapid population growth, elevated agricultural practices and the selective survival advantage of *P. falciparum* among African individuals (Cunha-Rodrigues et al. 2008).

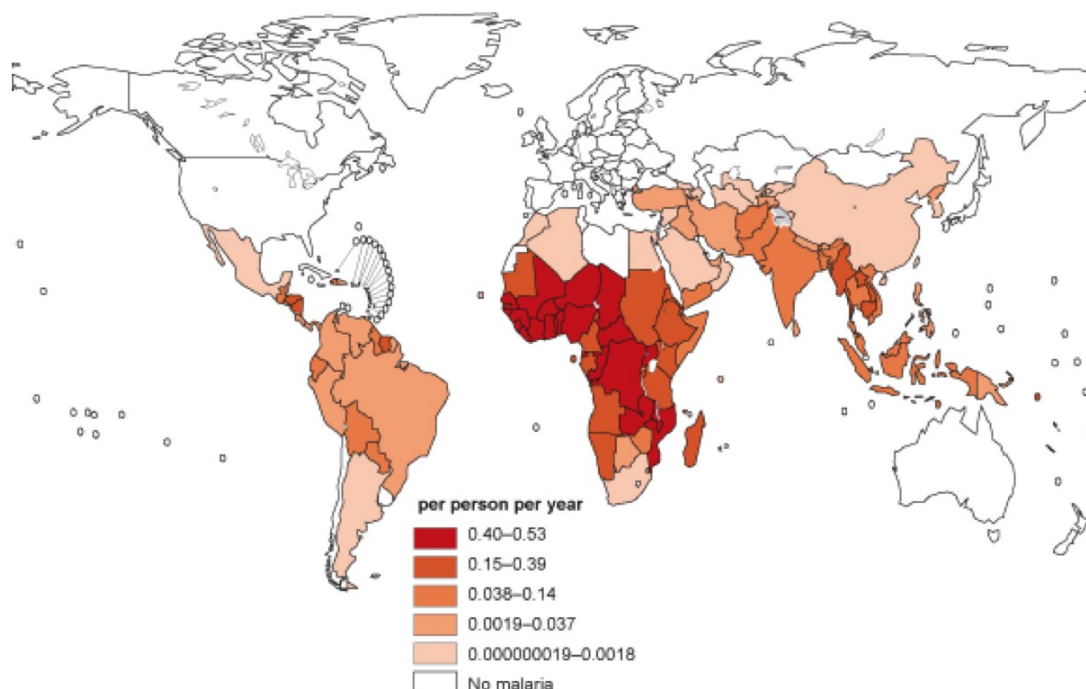


Fig. 1.3 Global distribution and burden of malaria in 2013

Most of the malaria cases are reported from African region. Countries from south-east Asia are reasonably affected by malaria infection. South American countries are also prone to malaria disease. Adapted from WHO world malaria report, 2014.

Over time, malaria spread to different parts of the world and today exhibits significantly different patterns of epidemics between regions and even within individual countries due to the variation in parasites and vectors, the difference in ecological dynamics and the variety of socioeconomic factors such as the degree of poverty and the efficiency of health care prevention systems. Most of the countries in East, Central and West Africa, Papua New

Guinea, the Solomon Islands and Vanuatu are considered as stable malaria endemic regions and some parts of Southern Africa, Transcaucasia, Central Asia and the Americas are acknowledged as unstable malaria settings (Fig. 1.3).

In 2014, reported clinical episodes of malaria were about 198 million with worldwide deaths estimated at 584,000 and located predominantly in sub-Saharan Africa. The incidence and mortality rates have decreased dramatically since 2000 due to increased interventions by 25% globally and 31% in Africa and by 42% globally and 49% Africa, respectively. The scale-up of prevention and control measures has protected an estimated 3.3 million lives worldwide between 2000 and 2012, most of which were young children (approx. 3 million).

Worldwide, malaria kills about 1,300 children every day, which amounts to approximately 482,000 children every year (World Health Organization 2014). Severe malaria, exclusively caused by *Plasmodium falciparum*, has adverse effects on pregnant women chiefly through severe anaemia, with at least 10,000 cases of malaria-related maternal mortality per year in sub-Saharan Africa (Omoti et al. 2013). Malaria infected pregnant women can also contribute to infant mortality by giving birth to underweight new-borns as a result of maternal anaemia and placental parasitaemia.

The malaria infection cycle:

Plasmodium parasites require both an invertebrate host (chiefly *Anopheles* mosquitoes) and a vertebrate host (e.g. human) for their sexual and asexual development, respectively, in order to complete their lifecycle successfully (Fig. 1.4). Both diploid and haploid forms of the parasite develop in the mosquito - the definitive host of the parasite - and only haploid forms are produced in humans. Female *Anopheles* mosquitoes ingest *Plasmodium* male and female gametocytes (sexual stages) during a blood meal, which soon develop into gametes that are then fertilised to form a zygote. The motile form of the zygote, called ookinete, crosses the gut epithelium about 24 h after a blood meal and transforms to an oocyst that remains attached to the basal site of the midgut. Sporozoites that develop inside the oocyst are released into the hemolymph when the oocyst bursts about 2 weeks later. They invade the salivary glands and are transmitted to a new human host through the saliva upon a subsequent mosquito bite. Once inside the host, sporozoites travel to the human liver where they begin their asexual cycle; they divide for 5-16 days inside hepatocytes to form tens of thousands of merozoites per cell. After this period, merozoites exit the liver and infect red blood cells where they replicate asexually every 1-3 days through repeated cycles of red blood cell invasion, replication and release, leading to the onset of illness. Instead of asexual replication, some merozoites transform into male and female gametocytes, which circulate in the blood stream awaiting for a mosquito bite (Ghosh et al. 2000).

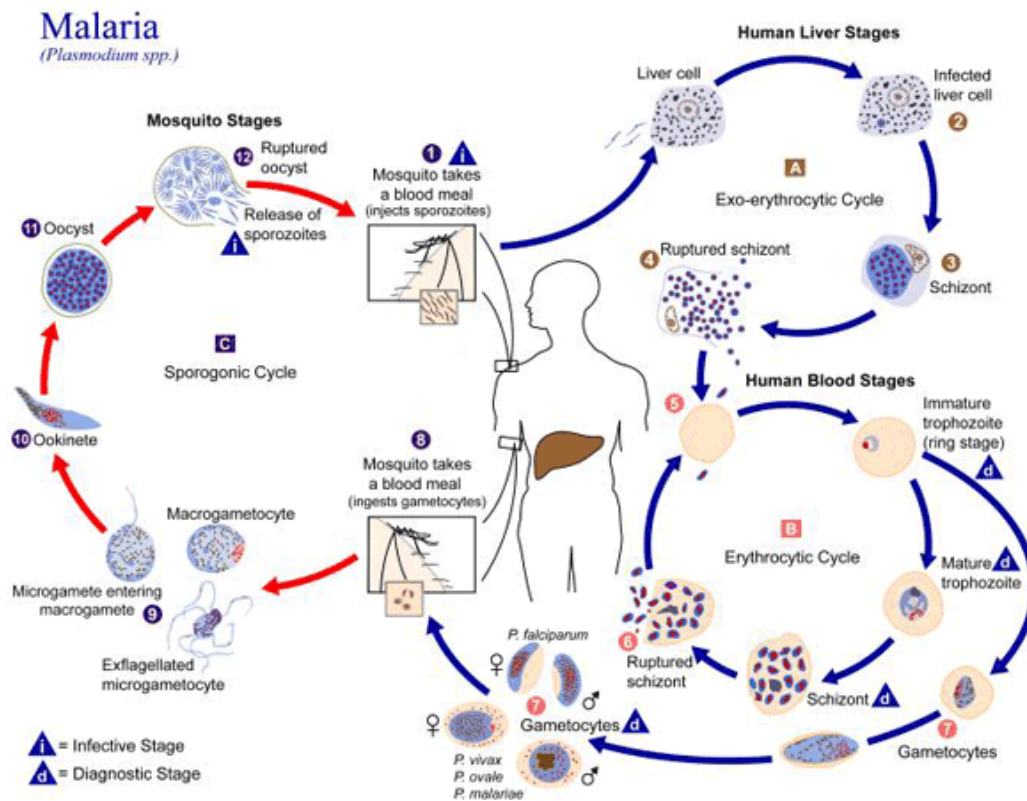


Fig.1. 4 The lifecycle of different malarial parasites in vertebrate hosts (Human) and in invertebrate hosts (mosquitoes)

Human, malaria parasites (sporozoites) grow and multiply asexually in the liver cells and in red blood cells (merozoites). Some merozoites form male and female gametocytes. During the blood feeding, mosquitoes pick up gametocytes from infected person. In the mosquito, malaria parasites complete their reproductive cycle and release sporozoites in the salivary glands. In vertebrate hosts, all developmental forms of parasites are haploid and in invertebrate hosts, parasites maintain both haploid and diploid forms. Source: www.cdc.gov.

There are about 3300 mosquitoes which have been documented globally to date. Chiefly, mosquitoes belong to the *Anopheles* genus, first described and named by J.W.Meigen in 1818, and are known to spread malaria from human to human. However, not all *Anopheles* mosquitoes recorded so far (around 400 species) transmit malaria. Variations in mosquito biology - collectively known as vectorial capacity - such as life span, inherent host preference for blood meal (human or other warm-blooded vertebrates), reproductive efficiency and susceptibility to parasitic developments change the ability/efficacy of Anopheline mosquitoes to carry and spread malaria. Only 70 *Anopheles* species are considered as potential vectors while 30 to 40 of are primary or effective vectors, which are distributed in different parts of the world (Figure.1.5).

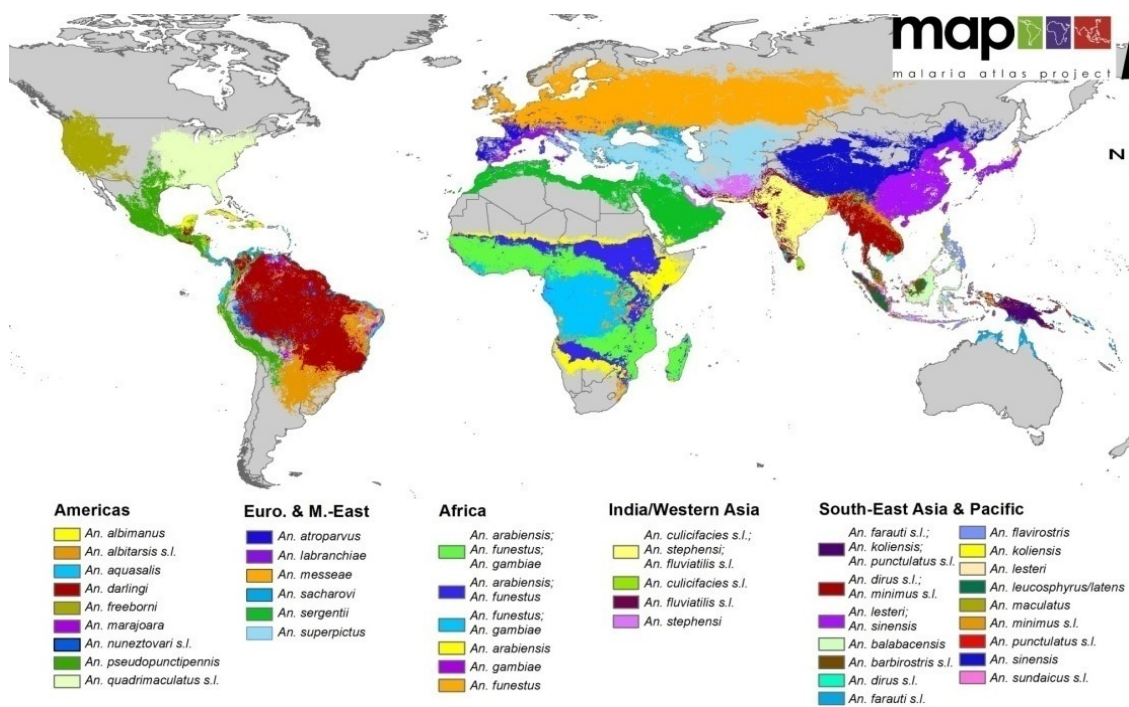


Fig.1. 5 Distribution of major malarial vectors (*Anopheles* species) in different parts of the world

Anopheles spp are found from most of the continents and each continent has different *Anopheles* spp complexes. SOURCE: Malaria Atlas Project.

Mosquito life cycle:

With a similar development to all other mosquitoes, *Anopheles* mosquitoes proceed from their eggs through to larval and pupal stages to the adult (Fig.1. 6). Adult males and females both feed on nectar and other sources of sugar for energy, and only females require blood meal from either human or other vertebrate hosts in addition, for the purpose of egg development. Due to this blood feeding behaviour, only female mosquitoes act as a malarial vector. In natural settings, males live for about a week and females survive no longer than one to two weeks. When most of the life span regulatory factors such as temperature, humidity and blood meal availability are controlled in a laboratory set-up, female mosquitoes can live longer than a month.

Eggs:

After few days of blood feeding, female mosquitoes lay eggs - approximately 50- 200 per oviposition - directly on water. These curved eggs are unique in having air-filled floats on either side. The eggs are non-resistant to desiccation and usually hatch within 2-3 days with

egg hatching time varying with temperature; for example, hatching may be delayed in winter or in cold climates.

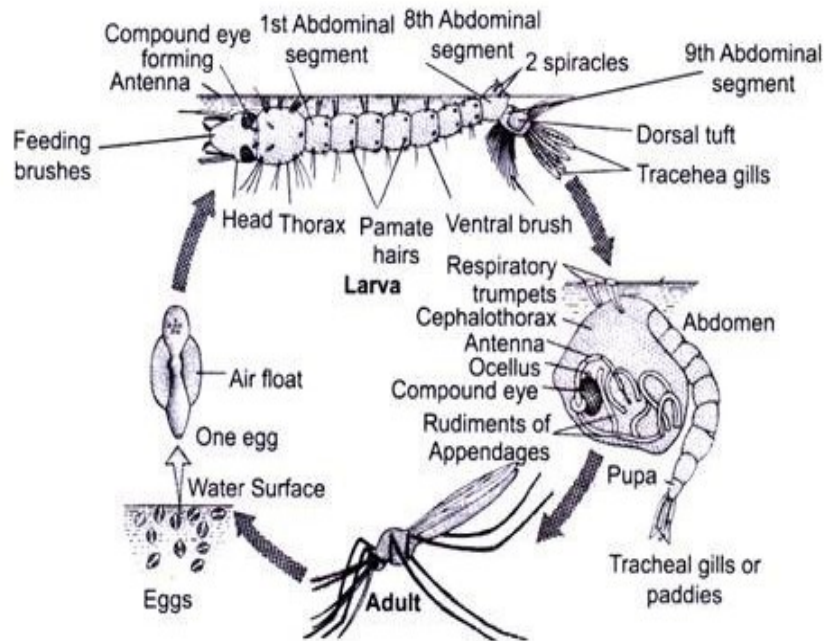


Fig.1.6 Life cycle of *Anopheles* species (Adapted from Climent, 1993)

Life cycle of mosquito comprises of eggs, larval, pupal and adult stages. Larva spends most of the time on feeding. In larval stage, they shed their skin and changes into pupa. Pupal stage is non-feeding stage. Adult mosquito has three parts of the body: head, thorax and abdomen specialized in, respectively, obtaining sensory information and feeding, locomotion and blood digestion and egg development.

Larval Stage:

The larval stage of *Anopheles* is completely aquatic and spends most of that time on feeding. Spiracles, located on the 8th abdominal segment, are specialized for breathing and mouth brushes on the head are specialized for feeding. This occasional cannibal larva feeds on microorganisms in the surface microlayer, such as algae and bacteria. Larvae of *Anopheles* species have been found in a wide range of habitats - freshwater or saltwater marshes, mangrove swamps, rice fields, and grassy ditches, the edges of streams and rivers, and small, temporary rain pools. Larvae metamorphose into pupae through four stages, called instar. In order to continue growing, the larvae moult, shedding their exoskeleton, or skin at the end of each instar (Baldini et al. 2014).

Pupal stage:

The comma-shaped, non-feeding pupal stage organism breath through a pair of respiratory trumpets on their cephalothoraces and adult mosquitoes emerge by splitting the dorsal surface of cephalothorax after 2-3 days of pupal stage development(Baldini et al. 2014).

Adult stage:

Development duration of *Anopheles* mosquito from egg to adult can vary from 5 to 14 days in different species. The slender-shaped adult female mosquito has three segments: head, thorax and abdomen specialized in, respectively, obtaining sensory information and feeding, locomotion and blood digestion and egg development (Fig. 1.7). Antennae and maxillary pulps are both highly modified for host and breeding site-odour detection. Male and female mosquitoes can be identified by their antennae size, with the male having a longer and bushier antenna than that of female mosquitoes. Adult mosquitoes usually mate within a few days of emergence. Female mosquitoes usually mate once in their lifetimes and preserve enough sperm in their body to fertilize a lifetime supply of eggs(Baldini et al. 2014).

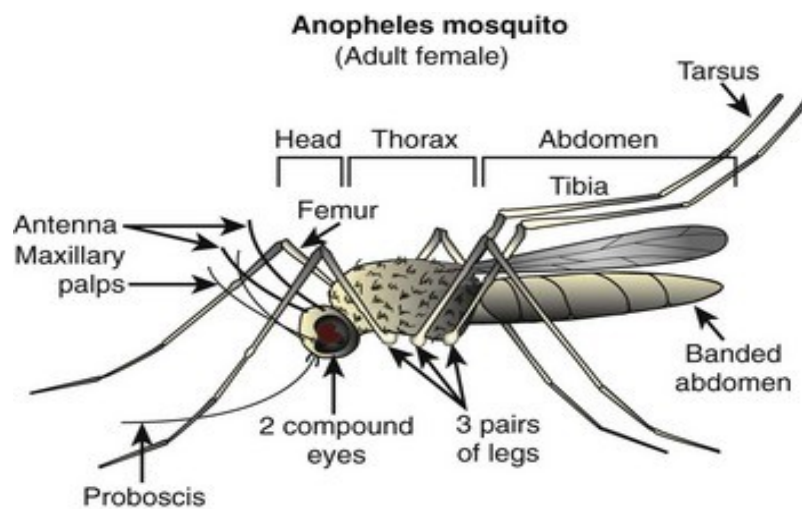


Fig.1. 7 Anatomy of the adult female *Anopheles* mosquito. (Adapted from clinicalgate.com)

The slender-shaped adult female mosquito has three segments: head, thorax and abdomen specialized in, respectively, obtaining sensory information and feeding, locomotion and blood digestion and egg development. Antennae and maxillary pulps are both highly modified for host and breeding site-odour detection. Male and female mosquitoes can be identified by their antennae size, with the male having a longer and bushier antenna than that of female mosquitoes.

VBD prevention and control

The global spread of VBDs is an increasing cause of death and suffering worldwide. Clinical management and control of VBD transmission are two major approaches within the framework of disease control tactics.

Management of clinical manifestations of VBDs focusing on malaria:

The available tools in combatting VBDS are prophylactic or therapeutic drugs (often the last line of defence/control of the disease) and vaccines. Since the efficiency of the drugs is dependent on the emergence of pathogen resistance, development of new drugs is essential. A wide variety of drugs is commercially available for some VBDs. Vaccinations are the safest, most affordable and effective method of preventing infectious diseases (Table 1.3).

Control of mosquito vectors:

Vector control is another fundamental part of the existing global strategy for the reduction or interruption of the transmission of VBDs, especially in mosquito borne diseases. Chemical, biological or genetic approaches, directed towards adult or larval stages, are being used to control mosquito populations, particularly in highly malaria prone areas. Recently, experts are emphasizing the operation of all possible prevention and control methods of VBDs simultaneously, rather than depending on a single method.

Core Vector control method:

Application of insecticides is longstanding and is the most important of the global mosquito-borne diseases management techniques. Significant scaling-up of insecticide usage around the world, owing to their efficacy and safety, reduced malaria incidences markedly in malaria endemic areas, especially in sub-Saharan Africa. Indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs) treated with pyrethroids are the two most effective and widely used mosquito control strategies which protect humans from the VBD infection by killing pathogen-carrying mosquitoes (Liu 2015). In the case of IRS, insecticides are being sprayed on the interior walls of houses in the most endemic areas, like Africa, where mosquitoes usually rest on the surface of house walls after a blood meal. During this resting time, it is possible that the insecticide on the wall kills mosquitoes infected with pathogens, such as malarial parasites.

Table 3. Clinical managements of the major VBDs

Disease	Regimen/Drugs	Vaccine
Malaria	Artemisinin-based combination therapy (ACT) particularly for <i>P. falciparum</i>	First vaccine designed for African children got clearance from The European Medicines Agency
Dengue(DEN)	No cure only supportive care	No available vaccine
Chikungunya(CHK)	No cure only supportive care	No available vaccine
Yellow Fever(YEF)	No specific treatment, only supportive care	The vaccine is safe, cost-effective and highly efficient.
Japanese encephalitis (JE)	Only supportive care is available.	Vaccines available privately.
Lymphatic filariasis	Multi-drug therapy : Albendazole (400 mg) together with Ivermectin (150–200 mcg/kg) or with Diethylcarbamazine citrate (DEC) (6 mg/kg).	No vaccine available for humans.
Rift Valley Fever(RVF)	No specific treatment for humans and other vertebrate hosts such as cattle, sheep and goats.	Vaccine available for vertebrate hosts, other than humans.
West Nile fever	Only supportive care.	Two kinds of vaccines are available - an inactivated WNV vaccine and a recombinant vaccine that uses canarypox virus to express WNV antigens.
Viseral Leishmaniasis	Pentavalent antimonials, Amphotericin B, Liposomal amphotericin B , Miltefosine and Paromomycin sulphate.	No vaccine for humans.

SOURCE: WHO, 2015; Hayes, 2005, Freitas-junior, 2012;OIE technical disease card, 2009

This is how IRS plays a key role in the reduction of disease transmission. There are 12 insecticides, in six classes - namely: organochlorines, organophosphates (OP), carbamates,

pyrethroids, pyrroles, and phenyl pyrazoles—which are recommended by the WHO for use against adult mosquitoes. Insecticides such as DDT, pyrethroids permethrin and deltamethrin are, therefore, widely used in malaria endemic areas to fight against malaria and also to slow down the evolution of insecticide resistance.

With approximately 5 years of trials, the use of LLIN has a validated track record of reducing or interrupting disease transmission twice-as-better compared to the untreated nets (Pates and Curtis, 2005). However, the cost of LLIN is still unaffordable for developing countries, especially for those most at risk.

Complimentary vector control method:

In a specific framework and under special circumstances, environmentally friendly biological controls can supplement the core vector control strategies and thereby avoid the ecological impacts of chemicals use. Since spores of entomopathogenic fungus *Beauveria bassiana* reduce mosquito populations by up to 80% in the laboratory they can be used as bio-adulticides (Blanford et al. 2005; Scholte et al. 2005) . Larval source is also possible through the destruction of vector breeding sites and by the use of larvivorous fish or bacterial larvicides like *Bacillus thuringiensis* (Mittal 2003) which can also be used to kill vector larvae.

Another environmentally non-polluting VBD control strategy is sterile insect technique (SIT). It is a gathering a lot of attention nowadays, owing to its species specific and effective results and partially due to the recognition of the limitations of current vector control strategies. Massive improvements in technologies like recombinant DNA technology, irradiation, aerial release and the use of gut microbiota i.e. *Wolbachia*, increase SIT's applicability and attractiveness (Alphey et al. 2010).

Insecticide resistance:

Mosquito resistance to chlorinate – a type of hydrocarbon insecticide - was first reported in the 1950s. Since then, widespread evolution of mosquito resistance to most of the commonly used insecticides is found to be making a significant contribution to the mortality and morbidity rates caused by VBD infections. Intensive selection pressure from insecticides is the main driver of the development of such resistance in the mosquito population. Two major, widely spread resistance mechanisms i.e. elevated metabolic detoxification activity and target site insensitivity, are involved in individual mosquito species (Liu 2015). A number of outbreaks related to mosquito-borne diseases in the recent past are the result of unchecked mosquito insecticide resistance (Hemingway 2002). All chief malarial vectors (*Anopheles* species) from 64 countries around the globe have shown resistance to all four existing classes of insecticides (World Health Organization 2012) . Therefore, it is crucial to

develop and implement tools to monitor resistance in endemic countries in order to control the emergence at new sites. Simultaneously, it is also important to develop new insecticides targeting new sites.

***A. coluzzii*, a new member of *A. gambiae* complex:**

Anopheles gambiae complex mosquitoes are distributed throughout the tropical Africa and considered as the major vector of malaria in Africa. This species complex consists of morphologically indistinguishable mosquitoes such as *A. gambiae*, *A. arabiensis*, *A. quadriannulatus* and *A. bwambiae*. In the past decade *A. gambiae* was split in M form (Mopti chromosomal form) and S form (Bamako/Savanna populations) on the basis of different inversion arrangements on the right arm of chromosome-2. The right arm of chromosome-2 are believed to facilitate high ecological flexibility and more efficient exploitation of different ecological niches, through the capture and stabilization within inversions of locally adapted genes (Ayala & Coluzzi 2005; Coluzzi et al. 1985). *A. gambiae*-M form prefers long-standing man-made breeding sites whereas *A. gambiae* –S form is associated with ephemeral and rain-dependent breeding sites (della Torre et al. 2005; Kamdem et al. 2012; Lehmann & Diabate 2008). SNPs analysis (400,000) across the genomes of paired population samples of M and S form suggested that the two taxa are evolving collectively on independent evolutionary trajectories (Reidenbach et al. 2012). Moreover, premating barriers present between M and S (Diabate et al. 2009; Pennetier et al. 2010). Based on molecular and bionomical evidence, the *An. gambiae* molecular "M form" is named *Anopheles coluzzii* Coetzee & Wilkerson **sp. n.**, while the "S form" retains the nominotypical name *Anopheles gambiae* Giles. X-chromosomal distributions of divergence between *A. coluzzii* and *A. gambiae* S.S form reveals a slight elevation in genetic divergence in a chromosomal region where nucleotide diversity is low relative to the rest of the chromosome (~16–20 MB) (Crawford et al. 2015). Indeed, this particular region coincides with a genomic region recently shown to play a role in assortative mating between these taxa (Aboagye-Antwi et al. 2015), further supporting its role in speciation, especially the early stages.

Metabolism of nutrients in the adult mosquitoes:

Plant sugars (such as nectar, fruit and other plant juices) and vertebrate blood are the major sources of nutrition in mosquitoes. Sugar ingestion, a principal characteristic of both sexes

and all ages of adult mosquitoes, provides energy for survivorship, flight, and enhanced reproduction. A blood meal that is taken exclusively by female mosquitoes is a vital metabolic process serving as a metabolic energy reserve in addition to its major role in egg development (Handel 1984). In unfavourable climatic and nutritional conditions, female mosquitoes supported by stored fat (derived from sugar and blood metabolites) can survive longer time compare to male mosquitoes. Like most of the insects, adult mosquitoes carry over energy reserves from their pupal stage. Mosquitoes from both sexes (male and female) contain smaller amount of fat during adult eclosion.

Sugar digestion and metabolism:

Constant sugar feeding is the essential part of the diet of adult mosquitoes. These insects obtain sugar from the nectar of flowers. Sugars of fruit juices and flower nectars consist mainly of glucose, fructose and sucrose with the absence or presence in trace of more complex sugars. Sugar meal metabolism in mosquitoes is advantageous for several metabolic needs, increasing life span, fecundity and available energy for flight (Grimstad & DeFoliart 1974; Handel 1984; Foster 1995). The sugar meal sustains the female mosquitoes until it finds its host and allows an infected mosquito to live long enough to oviposit, to bite repeatedly, and to become infective. Ingested sugar is temporarily deposited mainly in the large ventral diverticulum (crop) that is connected to the gut. In mosquitoes, sucrose digestion is carried out by the action of α -glucosidase enzymes in the midgut (Marinotti et al. 1996). α -glucosidases cleave sucrose and release α -glucose (Chiba 1997). Like other insects, mosquitoes absorb glucose from the gut and convert glucose to trehalose in the fat body and secrete into hemolymph. Trehalose is the chief hemolymph sugar in insects including mosquitoes (Handel 1984). Mosquitoes convert extra sugars to a somatic energy reserve in the form of a lipid-glycogen pool, mainly in the fat body (Mostowy & Foster 2004). Several studies have demonstrated that sugar feeding and digestion are found in all states of adult mosquito life, with the exception of its slight inhibition during and soon after blood meal digestion by female mosquitoes (Foster 1995). Moreover, fat content increases steadily in female mosquitoes and completely disappears in male mosquitoes when they are maintained only on sugar after eclosion.

Blood meal digestion and metabolism:

Blood feeding is a unique trait of hematophagous mosquitoes. They accumulate nutrients from the blood for egg development. They also obtain somatic energy reserve like lipid and glycogen from blood meal. The ingested blood meal is directly taken in the mosquito midgut. In the posterior midgut of a female mosquito, the ingested blood is digested into amino acids, chiefly by the activity of blood meal-induced trypsin-like proteinase enzymes. A.

gambiae has seven trypsin genes and two of these Antryp1 and Antryp2, have shown to be expressed in the mosquito midgut following a blood meal (Müller et al. 1993). Initially, mosquitoes release haemoglobin, the major blood protein, by breaking down the membranes of red blood cells (Gaio et al. 2011). Mosquitoes use amino acids for protein synthesis and also for fatty acid biosynthesis from reduced carbons derived from amino acids (Alabaster et al. 2011). 65% of labelled amino acids derived from blood meal were fully oxidized or excreted, 15% was found in maternal and egg lipids, 10% was converted to egg proteins and remaining was distributed amongst glycogen and other metabolites in *Ae. aegypti*. Haemoglobin provides high level of iron which is crucial for egg development and viable offspring (Zhou, Flowers, et al. 2004). Most of the hematophagous arthropods like mosquitoes get sufficient cholesterol from their diet which serves as structural component of cell membranes. This cholesterol also serves as the precursor of the insect molting hormones and ecdysteroid (Estela L Arrese & Soulages 2010).

Lipolysis, absorption and export:

Dietary TAGs convert into monoacyl-glycerols and free fatty acids in the midgut lumen in the presence of lipases that produced by midgut cells. These free fatty acids enter into midgut cells and convert into diacylglycerol (DAG), triacylglycerol (TAG), and phospholipids in most of the insects. It is possible that phosphatidic acid pathway and the monoglycerol pathway may involve in the synthesis of DAGs and TAGs. Dietary cholesterol mainly absorbs in the midgut (Canavoso et al. 2001).

Insects can either export DAG to the hemolymph or can rapidly convert to TAGs which can serve as a reservoir for absorbed fatty acids. DAG is the major lipid circulating in the hemolymph after lipid digestion. Lipophorin (LP) picks up DAG and delivered to fat body, central reservoir for excess nutrient. It is notable that lipophorin does not enter the midgut cells or fat body (Sun et al. 2000).

Lipophorin, the major hemolymph lipoprotein, transports lipid throughout the insect body, by loading and unloading into and from the sites of their utilization (i.e muscle, ovary) and storage (i.e fat body). Insect Lp transports mainly DAG and phospholipids. Lp consists of two apolipoproteins- apoLpI and apoLpII. Another small apolipoprotein, apoLp III, is associated with Lp in a reversible manner (Blacklock & Ryan 1994; Kawooya et al. 1986; Surholt et al. 1992; Soulages & Wells 1994; Van der Horst 1990). HDLp receives DAGs from the fat body in response to a stimulatory signal such as the high energy requirement in the body. This loading of lipid transforms HDLp into low-density Lipoprotein (LDLp). During this transformation several ApoLpIII come to add with LDLp. LDLp unloads lipid into the delivery

sites such as muscles and ovaries of mosquitoes. At the same time it releases ApoLpIII and transforms itself back to HDLp(Gondim et al. 1992; Shapiro et al. 1988).

Fat storage in fat body:

The insect fat body is a multifunctional organ which stores lipid, and glycogen, synthesizes hemolymph proteins, and also helps in metabolism. It is a loose tissue and is bathed by the hemolymph. This maximal exposure of fat body to the hemolymph helps the organism to cope up with the changes in the concentration of energy precursors in circulation. Fat body is mainly made up of adipocyte, characterized by the presence of numerous lipid droplets(Estela L. Arrese & Soulages 2010).

Lipid droplets are specialized cytoplasmic compartments for intracellular storage of TAG. Although TAG can be stored in small lipid droplets in almost all tissues, adipocytes, the basic cells of the fat body, are specialized for lipid storage (Estela L Arrese & Soulages 2010). Lipid droplets are composed of a core of TAG and cholesterol esters enclosed by a monolayer of phospholipid and cholesterol. Specific proteins are either embedded in or peripherally attached to the lipid droplets. Lipid droplets are synthesized after feeding and occupy most of the intracellular spaces, along with glycogen and other protein granules (Bickel et al. 2009; Bowen 1992).

Fatty acids are promptly taken up by the fat body and are readily incorporated, chiefly into TAG and, in small amounts, into other glycerides and phospholipids. This loading and unloading of lipids into and from fat body is performed by a shuttle mechanism which involves LP and lipid transfer molecules. The rate of assimilation of these fatty acids and acetate into the fat body is reliant on the developmental stage and feeding status of the individual. For instance, in female *Ae. aegypti*, 50% of all glucose derived from their diet is used for the synthesis of lipids, while 35% is involved in glycogen synthesis (Zhou, Pennington, et al. 2004). Most of the glycogen provides energy to post-feeding larval stages and the rest is carried over into pupae and adults, whereas lipid stores remain stable during larval life and are preserved to be used by both pupae and adults (Estela L Arrese & Soulages 2010).Egg development depends on the substantial mobilization of lipids from the fat body to the ovaries.

Lipid delivery to ovarian tissues:

Oocyte development in insects involves accumulation of a large amount of extra-ovarian lipid delivered by LP. Insects synthesize 1% of the lipid of the egg and another 5% of the lipid is delivered by the yolk protein vitellogenin. There are two mechanisms are involved in the LP-mediated lipid delivery into developing oocytes. Most of the lipid(DAG) (90% of total lipid) is

delivered by HDLp, however, some of the lipid is delivered to the egg by HDLp through selective endocytosis of HDLp. Intracellularly HDLp stripped of most of its lipid and stored in the developing egg as very high-density lipoprotein (VHDLp)(Kawooya et al. 1986).

Steroyl Co A desaturase enzyme: Rate limiting enzyme in fatty acid metabolism:

TAGs are synthesized through a step-wise conversion of the key substrate of fatty acids, known as acetyl CoA (Fig.1.8). In the terminal, and only committed step of TAG formation, diacylglycerol (DAG), which is composed of two fatty acid and one small carbohydrate glycerol molecules, is bonded with acetyl co-A through ester linkage in the presence of a diacylglycerol acyltransferase enzyme.

Fatty acids, the building blocks of TAG and other fats, are carboxylic acids that consist of an aliphatic tail and a terminal carboxyl group. They are either saturated (SFA) or unsaturated (UFA) and most of the natural fatty acids have a chain of carbon atoms ranging from 12 to 28. Fatty acids are of crucial biological importance as they are involved in energy storage, signal transduction and maintenance of cell membrane fluidity (Athenstaedt & Daum, 2006; Fahy et al., 2008).

The introduction of the first double bond into SFAs at the delta 9 position (between carbons 9 and 10) is the most critical commitment step in the biosynthesis of mono unsaturated fatty acids (MUFAs). Stearoyl-CoA desaturase (SCD) is an iron containing, microsomal enzyme conserved in all eukaryotes, which catalyses this reaction that additionally requires cytochrome b5, NADH (P)- cytochrome b5 reductase, and molecular oxygen (Ntambi 1999). Major MUFAs, such as oleic acid and palmitoleic acid, are key precursors of membrane phospholipids, cholesterol esters and triglycerides, and are synthesized by the SCD from SFAs such as stearic acid and palmitic acid, respectively (Ntambi 1999). SCD is considered a pivotal enzyme in the body, since the ratio of stearic acid and oleic acid plays an important role in the maintenance of cell membrane fluidity and cell-cell interactions.

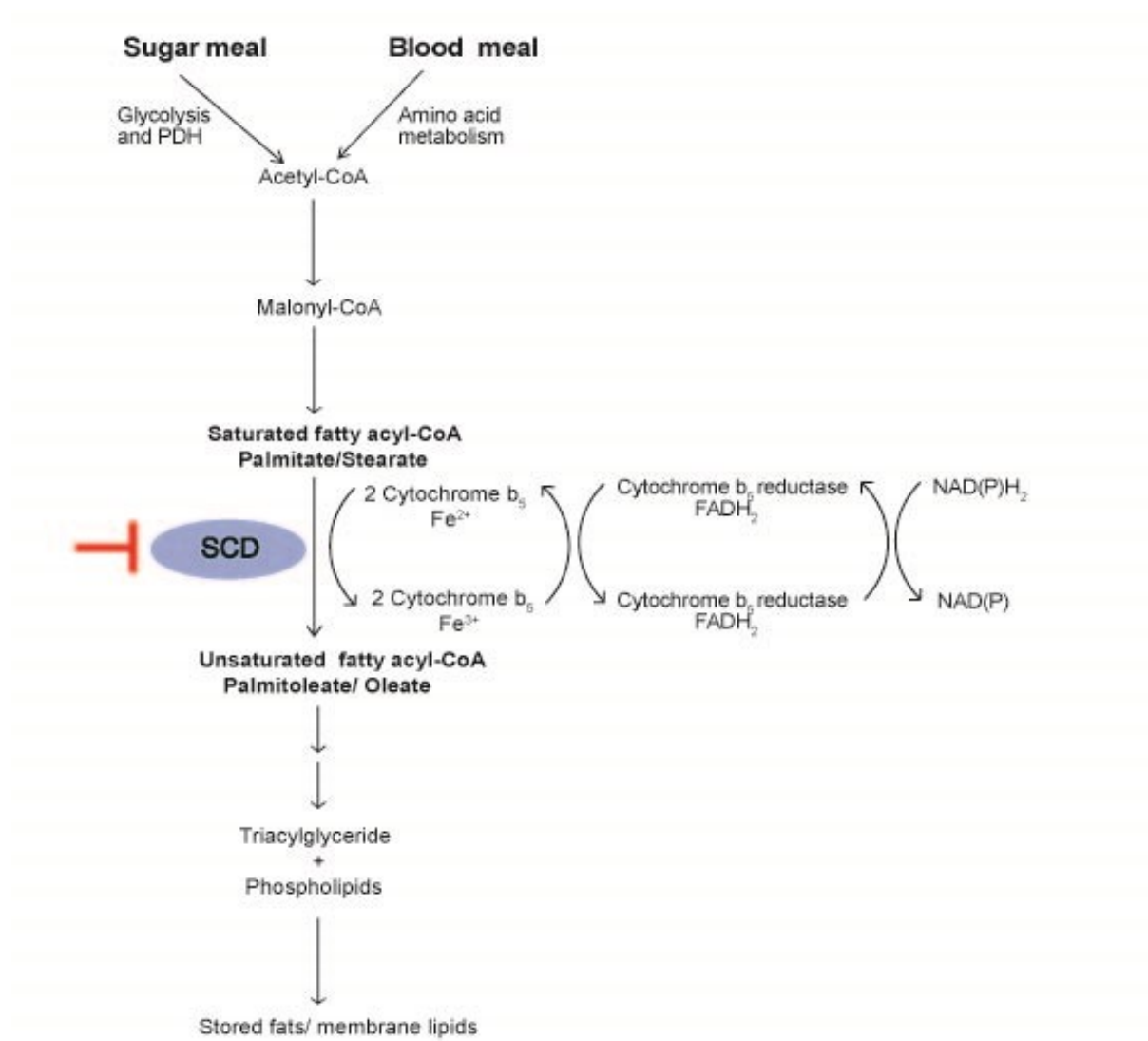


Fig. 1.8 Overview of the fatty acid biosynthesis pathway in insects.

The schematic is modified from Alabaster, 2011. Hexose sugars from nectar meals, and ketogenic amino acids from blood meals, convert into the fatty acid substrate acetyl-CoA, which is used to synthesize malonyl-CoA, the precursor to palmitate. SCD1 enzyme catalyzes the reaction where SFAs (i.e palmitate), converts into UFA, key precursor of triacylglycerols and phospholipids

It has been shown that atypical alteration of the tightly maintained ratio of fatty acids in the human body through the inhibition of SCD function can lead to several disorders such as diabetes, cardiovascular disease, obesity, hypertension, neurological diseases, immune disorders, cancer, and aging (Ntambi 1999). In insects, SCDs are shown to play essential roles in lipid metabolism, maintenance of the cuticular hydrocarbon structure and preserving the integrity of the structure and function of biological membranes (Roelofs & Rooney 2003; Howard & Blomquist 2005).

Several SCDs have been identified and characterized so far in mammals; two SCD homologues (SCD1 and SCD5) in humans and four (SCD1-SCD4) in mice (Bai et al. 2015). The expression of the *SCD1* gene is regulated by a number of factors including dietary, hormonal and/or environmental. It is triggered by a carbohydrate-heavy diet, including glucose and fructose, cholesterol, and vitamins A and D, while it is inhibited by high concentrations of poly-unsaturated fatty acids (PUFA) and linoleic acid. Several transcription factors, such as liver X receptor, sterol response element binding protein 1c, and carbohydrate response element binding protein regulate *SCD1* expression.

In female mosquitoes, the only available data suggest that *SCD1* expression is highest in the carcass and midgut, followed by the head, salivary glands, malpighian tubules and ovaries (Marinotti et al. 2005). In male mosquitoes, the highest levels of expression are seen in the carcass followed by the head, salivary glands, malpighian tubules, testes and midgut. Therefore, female mosquitoes exhibit significantly higher transcriptional expression of *SCD1* in the midgut compared to male mosquitoes.

SCD1 inhibitors:

Sterculic oil (SO) is the most recognized natural inhibitor of stearoyl coA desaturase. It inhibits oleate formation (18:1) through the desaturation of exogenous stearate (18:0) in prokaryotic and eukaryotic cell lines, and cholesterol biosynthesis (Zoeller & Wood, 1985; Wältermann & Steinbüchel, 2000). It also causes alterations in the permeability of the cell membrane and cell division (Lam N Nguyen et al. 2014). SO, usually extracted from plant sources such as the seeds of the wild almond tree *Sterculia foetida* and the seeds of the common cotton plant *Gossypium hirsutum*, contains two cyclopropenoic fatty acids, sterculic acid (SA, 55%) and malvalic acid (10%) (Ortinou et al. 2012). SA and malvalic acids are also potent inhibitors of delta (9) desaturation in various C-12 to C-20 aliphatic acids (Hernando et al. 2002). Sterculic acid was first discovered by Nunn in 1952 from *sterculia foetida* oil and named by Schlenk. The chemical names of sterculic acid and malvalic acids are 9, 10-methylene-9-octadecenoic acid and 7-(2-octyl-1-cyclopropenyl) heptanoic acid, respectively (Fig. 1). Both *in vitro* and *in vivo* studies have confirmed that the fat composition-manipulating ability of SO is due to a reduction of *SCD1* activity (Ortinou et al. 2012). However, the exact mechanism of this inhibition of the *SCD1* enzyme by SA is still not clear (Wältermann & Steinbüchel 2000).

Recent studies support the use of SCDs as potential chemotherapeutic targets for the treatment of various metabolic disorders and infectious diseases (Igal 2011). In the development of a new drug, it is crucial to understand the kinetics of active metabolite formation, in order to predict the therapeutic outcome and to explain the toxicity of specific

drugs (Nixon et al. 1977). It has been shown that, labelled SA is absorbed and metabolized at a faster rate in the Wistar rat model when administered by intragastric intubation rather than intraperitoneal injection. The concentration of labelled SA reaches maximum levels in blood serum 2 h after intubation and then drastically drops. At 4 h after intubation, SA accumulates in different organs, with the concentration in the liver peaking at the maximum administered doses. SA is excreted mainly in the faecal matter (48% in urine and 11% in faeces), with a low recovery of labels in the excreted CO₂ at 16h post-intubation (Nixon et al. 1977).

Chapter 2

Aims and Objectives

The mosquito *Anopheles gambiae*, now divided into two sibling species, *A. gambiae* and *A. coluzzii*, is the main vector of malaria in sub-Saharan Africa. In the past 15 years, largely due to genome sequencing, this mosquito has become an important model organism to study vector-parasite interactions and disease transmission. Advances in the understanding of its biology have improved our capacity to manipulate this mosquito and develop strategies to halt malaria transmission. In this PhD project, the function of the *A. coluzzii* stearoyl CoA desaturase1 (SCD1), a critical regulator of lipid metabolism and other physiological processes related to blood feeding has been investigated with the goal of contributing to the development of novel disease control interventions. Prior to the start of this thesis, preliminary data in the host laboratory suggested that SCD1 might be essential for mosquito survival following a blood meal.

The specific objectives of this thesis were to:

Dissect the function of SCD1 in blood meal metabolism and mosquito physiology using a gene silencing approach.

Provide insights into the genetic and metabolic pathways affected by SCD1 by profiling the transcriptomes and metabolomes of SCD1 loss-of-function mosquitoes.

Exploit the function of SCD1 towards development of novel vector and disease control interventions.

Chapter 3

Phenotypic characterization of the Stearoyl-CoA desaturase 1 (*SCD1*) gene in *A. coluzzii* mosquitoes

Summary

Fatty acid $\Delta 9$ -desaturase activity is required for the conversion of saturated (SFAs) to mono-unsaturated (MUFAs) fatty acids that serve as precursors of major structural components of the cell membranes and are essential for survival, reproduction and blood meal digestion in adult female *Anopheles* mosquitoes. Here, the *A. coluzzii* Stearoyl-CoA desaturase 1 (*SCD1*), encoded by the VectorBase gene AGAP001713, is phenotypically characterized through a series of experiments that involved RNAi-mediated gene silencing in adult female mosquitoes. Compared to control mosquitoes, the mortality rate of *SCD1* KD mosquitoes was higher during sugar feeding and increased dramatically immediately after a blood meal; all mosquitoes died by 48 h post blood meal. Microscopic observations revealed that the body cavity of dead *SCD1* KD mosquitoes was filled with blood suggesting that the midgut epithelial integrity was severely compromised. Transmission electron microscopy showed that the midgut cell membranes are thick and rigid, and fail to extend and thin out after a blood meal. This is consistent with the homeostatic function of *SCD1* in maintaining an optimum ratio of SFAs to MUFAs, which is vital for cell membrane fluidity and plasticity. In addition, midgut cells of *SCD1* KD mosquitoes contained fewer, indistinct and irregularly shaped mitochondria, presumably due to altered energy metabolism and cell depolarisation; it is known that mitochondrial morphology and physiology are sensitive to the SFA/MUFA ratio. Finally, midgut cells of *SCD1* KD mosquitoes showed a substantial depletion of lipid droplets after a blood meal, the main energy and lipid storage source, while ovaries did not develop and remained small in size. These observations provide critical information for the understanding of lipid metabolism in *Anopheles* mosquitoes following a blood meal and the function of *SCD1*. They also provide important leads for the design and development of novel vector control interventions based on lipid metabolism after a blood meal.

Introduction

Nutrient metabolism:

Plant sugars (such as nectar, fruit and other plant juices) and vertebrate blood are the major sources of nutrition in mosquitoes. Sugar ingestion, a principal characteristic of both sexes and all ages of adult mosquitoes, provides energy for survivorship, flight, and enhanced reproduction. A blood meal that is taken exclusively by female mosquitoes is a vital metabolic process serving as a metabolic energy reserve in addition to its major role in egg development (Handel 1984). The obligatory requirement of female mosquitoes for a blood meal for nourishment of their eggs and for their reproductive fitness defines the role of mosquitoes in disease transmission (Zhou, Pennington, et al. 2004). During blood feeding, female mosquitoes ingest parasites (as well as other human pathogens such as arboviruses), which they can in turn be transmitted to other human hosts during subsequent blood meals.

Sugar Metabolism

Ingested sugar is temporarily deposited mainly in the large ventral diverticulum (crop) that is connected to the gut. It is gradually released and subsequently absorbed in the midgut where it accomplishes the mosquitoes' instant metabolic needs and maintains the trehalose pool of the hemolymph. Mosquitoes convert extra sugars to a somatic energy reserve in the form of a lipid-glycogen pool, mainly in the fat body, which is analogous to the adipose tissue and the liver in vertebrates, and in the thoracic muscles, (Mostowy & Foster 2004).

Blood metabolism and egg development

The ingested blood meal is directly taken in the mosquito midgut. In the posterior midgut of a female mosquito, the ingested blood is digested into amino acids, chiefly by the activity of blood meal-induced trypsin-like proteinase enzymes. Initially, mosquitoes release haemoglobin, the major blood protein, by breaking down the membranes of red blood cells (Gaio et al. 2011).

Blood feeding mosquitoes use blood meals to improve their energy status, predominantly in preparation for reproduction, as substantial lipid mobilization by lipophorin (Lp) from the fat body to the ovaries is required for egg development (Estela L Arrese & Soulages 2010). Lipid stores in insect oocytes are almost 30-40% of the female mosquito dry weight and are the principal source of energy for the developing embryo. Apart from the incorporation of lipids from exogenous sources, oocytes can also synthesize, de novo, more than 1% of total lipids (Arrese & Soulages, 2010). The direct precursor for triglyceride (TAG) synthesis in

oocyte is diglycerides (DAG). Usually, in a short period of time during their maturation (1-2 days), oocytes elevate their lipid content several fold, and mosquitoes enhance energy reserves for oocyte development by supplementary blood meals during the gonotrophic cycle (Clifton & Noriega, 2012). Since blood protein is singularly important for egg development, digestion of blood is positively correlated with the numbers of eggs produced. However, despite this correlation, increasing meal size decreases the conversion rate of amino acids to yolk protein (Gaio et al., 2011).

In the yellow and dengue fever mosquito *Ae. aegypti*, 10% of the amino acids ingested via a blood meal are directly assimilated into eggs, while 19% of amino acids are deaminated to replace the lipids carried to the oocytes from the fat body. The resulting portion of the blood meal is utilized to produce energy (43%) and waste material (29%) that is eliminated from the body (Isoe, Rascón, Kunz, & Miesfeld, 2009; Zhou, Pennington, & Wells, 2004; Zhou, Flowers, et al., 2004).

During blood feeding, dietary lipids are transported from the midgut by Lp, the major hemolymph lipoprotein composed of two apolipoproteins (apoLpI and apoLpII), to the metabolic and storage sites such as the fat body and also to utilization sites such as muscles (Sun et al. 2000). Mosquito Lp contains different neutral lipids such as Triacyl glyceride (TAG) that makes up 41% of total neutral lipids along with 32% phospholipids. It serves as a reusable lipid shuttle and does not enter into midgut cells or the fat body during this lipid loading and unloading process (Sun et al. 2000).

Fat storage and lipid droplets

TAG is the principal form (more than 90%) of the stored lipid in the fat body cells of mosquitoes. Culicomorpha (mosquitoes, black flies, midges) also convert carbohydrates to lipids through an inefficient process, known as *de novo* lipogenesis, and store them in the fat body (Estela L. Arrese & Soulages 2010). Somatic reserves of lipids and glycogen, which are synthesized from amino acids, fatty acids and carbohydrates, are used to support flight, survival, synthesis of storage proteins for metamorphosis, and vitellogenesis and synthesis other yolk proteins for egg development; Lp is then used to transport these lipids to the body. Among these two kinds of energy reserves, anhydrous and reduced TAG have caloric energy stores with a higher concentration than that of hydrous glycogen (Estela L. Arrese & Soulages 2010).

Lipid droplets are specialized cytoplasmic compartments for intracellular storage of TAG. Although TAG can be stored in small lipid droplets in almost all tissues, adipocytes, the basic cells of the fat body, are specialized for lipid storage (Estela L. Arrese & Soulages 2010).

Lipid droplets are composed of a core of TAG and cholesterol esters enclosed by a monolayer of phospholipid and cholesterol. Specific proteins are either embedded in or peripherally attached to the lipid droplets. Lipid droplets are synthesized after feeding and occupy most of the intracellular spaces, along with glycogen and other protein granules (Bickel et al. 2009; Bowen 1992).

Fatty acids are promptly taken up by the fat body and are readily incorporated, chiefly into TAG and, in small amounts, into other glycerides and phospholipids. The rate of assimilation of these fatty acids and acetate into the fat body is reliant on the developmental stage and feeding status of the individual. For instance, in female *Ae. aegypti*, 50% of all glucose derived from their diet is used for the synthesis of lipids, while 35% is involved in glycogen synthesis (Zhou, Pennington, et al. 2004). Most of the glycogen provides energy to post-feeding larval stages and the rest is carried over into pupae and adults, whereas lipid stores remain stable during larval life and are preserved to be used by both pupae and adults (Estela L Arrese & Soulages 2010).

Building blocks of TAG

TAGs are synthesized through a step-wise conversion of the key substrate of fatty acids, known as acetyl CoA. In the terminal, and only committed step of TAG formation, diacylglycerol (DAG), which is composed of two fatty acid and one small carbohydrate glycerol molecules, is bonded with acetyl co-A through ester linkage in the presence of a diacylglycerol acyltransferase enzyme.

Fatty acids, the building blocks of TAG and other fats, are carboxylic acids that consist of an aliphatic tail and a terminal carboxyl group. They are either saturated (SFA) or unsaturated (UFA) and most of the natural fatty acids have a chain of carbon atoms ranging from 12 to 28. Fatty acids are of crucial biological importance as they are involved in energy storage, signal transduction and maintenance of cell membrane fluidity (Athenstaedt & Daum, 2006; Fahy et al., 2008).

Stearoyl-CoA desaturase enzyme: A rate limiting enzyme

The introduction of the first double bond into SFAs at the delta 9 position (between carbons 9 and 10) is the most critical commitment step in the biosynthesis of mono unsaturated fatty acids (MUFAs). Stearoyl-CoA desaturase (SCD) is an iron containing, microsomal enzyme conserved in all eukaryotes, which catalyses this reaction that additionally requires cytochrome b5, NADH (P)- cytochrome b5 reductase, and molecular oxygen (Ntambi 1999). Major MUFAs, such as oleic acid and palmitoleic acid, are key precursors of membrane phospholipids, cholesterol esters and triglycerides, and are synthesized by the SCD from

SFAs such as stearic acid and palmitic acid, respectively (Ntambi 1999). SCD is considered a pivotal enzyme in the body, since the ratio of stearic acid and oleic acid plays an important role in the maintenance of cell membrane fluidity and cell-cell interactions.

It has been shown that atypical alteration of the tightly maintained ratio of fatty acids in the human body through the inhibition of SCD function can lead to several disorders such as diabetes, cardiovascular disease, obesity, hypertension, neurological diseases, immune disorders, cancer, and aging (Ntambi 1999). In insects, SCDs are shown to play essential roles in lipid metabolism, maintenance of the cuticular hydrocarbon structure and preserving the integrity of the structure and function of biological membranes (Roelofs & Rooney 2003; Howard & Blomquist 2005).

The importance of lipid metabolism in blood seeking insects offers an opportunity for exploitation towards development of novel vector control tools. Despite the conserved role of SCD1 in lipid biosynthesis (Ntambi 1999), there is a lack of data about the role of this enzyme during blood feeding and the biological processes regulating its activity. The aim of the experiments described here was to examine the phenotypic effects following silencing of the *SCD1* gene in *A. coluzzii* (formerly known as *A. gambiae* M form) mosquitoes.

Methods

Anopheles mosquito colony and maintenance

Female *A. coluzzii* mosquitoes of the Ngousso colony (established from field M molecular form and the Forest chromosomal form *A. gambiae* mosquitoes collected in Cameroon in 2006) were used in all the experiments. Adult mosquitoes were held in a netted cage and maintained on 5% fructose solution. The *A. coluzzii* colony was maintained in the insectary at a temperature of 28⁰ C (± 1 °C), 70-80% relative humidity and a 12 hour dark/ light cycle. Larvae were fed on tetramin fish food (Tetra, Germany).

Bioinformatics analysis

Genome searches against the *A. coluzzii* stearoyl-CoA desaturase (*SCD1*) gene (gene accession number AGAP001713) and other orthologs (Human, *Drosophila melanogaster*, *Plasmodium falciparum*) were conducted using the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI) and Vector Base (Lawson, 2012). Sequence alignments were performed using ClustalW multiple alignment (http://npsa-pbil.ibcp.fr/cgi-bin/align_clustalw.pl) and protein prediction was done by SMART Package (<http://smart.embl-heidelberg.de/>).

dsRNAs preparation and gene silencing

Gene-specific double-stranded RNA (dsRNA) was designed, synthesized, and injected as described by (Blandin et al. 2002). In brief, a target sequence was retrieved from VectorBase and appropriate gene-specific oligonucleotide primers were designed with a T7 RNA polymerase promoter sequence on the 5' end to produce dsRNA. RNA was extracted from 10 non-injected, sugar fed, whole, adult female mosquitoes using the TRIzol reagent (Invitrogen). Following the manufacturer's instructions of T7 MEGAscript kit (Invitrogen), dsRNAs for the target gene (*dsSCD1*) or the control (*dsLacZ*) were synthesized and further purified using the *RNeasy* kit (QIAGEN). Gene silencing was accomplished by injection of 69 nL (3 µg/µL) of *dsRNA* into the mesothoracic spiracle of each carbon dioxide-anesthetized female mosquito (0–2-day old). Three days were allowed for mosquito recovery. Primer sequences against *SCD1* are as follows with T7 tags identified with lower cases: *SCD1* RNAi For: taatacgaactcactataggg AAATGTGATTGCCTTCGGT; *SCD1* RNAi Rev: taatacgaactcactataggg GCGAGAAGAAGAAGCCAC. The primer against LacZ has been reported elsewhere (Stathopoulos et al. 2014)

RNA extraction and qRT PCR

In summary, total RNA was extracted from 10 whole (*dsSCD1/dsLacZ* - treated) mosquitoes per sample. cDNA synthesis was performed from total RNA following the manufacturer's instructions for the QuantiTect Reverse Transcription kit (QIAGEN). The gene silencing efficiency was determined using the SYBR *Premix Ex Taq* kit (Takara) according to the manufacturer's instructions and the ABI Prism 7500 Fast Real-Time thermocycler (Applied Biosystems). *Anopheles* ribosomal gene *S7* was used as the endogenous reference, and *SCD1* gene expression was quantified relative to a calibrator control sample (e.g. *dsLacZ* - Injected mosquitoes). qRT primers for the *SCD1* gene are For- GGTGTCTGAAGGAGATCGTGG / Rev- TCTGGTTGAGAATGGTGGCG and primers for *S7* have been reported previously (Habtewold et al. 2008)

Transmission electron microscopy

For Transmission Electron Microscopy (TEM), midguts from *dsLacZ* or *dsSCD1*- injected *A. coluzzii* females were dissected just before blood feeding (0h) and 24 h PBM through a membrane feeder device. Dissected midguts were fixed for 2 h at room temperature (2.5% E.M. Grade Glutaraldehyde, 0.1 M sodium cacodylate buffer containing 2 mM calcium chloride), transferred to 1% osmium tetroxide for 1 h, washed in deionized water, and stained with 2% aqueous uranyl acetate for 30 min. Specimens were dehydrated with ethanol dilutions, infiltrated with Spurr's resin, and flat-embedded at 60 °C. Transverse sections (70 nm) were prepared using a Leica ultracut UCT ultramicrotome and placed onto

200 thin mesh bar copper grids followed by counterstaining with lead citrate. Midgut sections were viewed with a JEOL-JEM-1230 electron microscope operated at 80 kV and TIFF images (8 bit) were captured using an AMT 4M pixel camera.

Results

Identification of a putative *A. coluzzii* Stearoyl CoA desaturase

Bioinformatics searches of the *A. gambiae* and *A. coluzzii* genomes revealed the presence of a gene (AGAP001713) encoding a putative stearoyl-CoA Δ^9 -desaturase on chromosome 2. The gene contains 5 exons and encodes a 355 amino acid-long protein. This protein has central FA_desaturase domain (68-309 aa) and a single transmembrane domain (Fig.3.1).

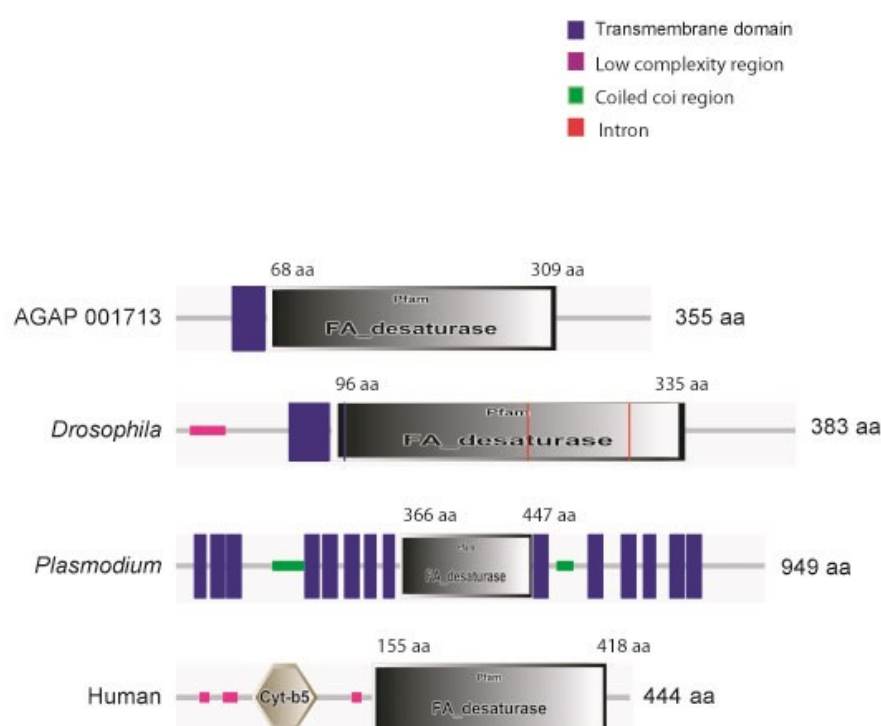


Fig.3.1 Scheme of the SCD1 protein of *A. coluzzii* and similarity analysis of orthologues in *D. melanogaster*, *P. falciparum* and human

SCD1 proteins of *A. coluzzii*, *D. melanogaster*, *P. falciparum* and human contain 355, 383, 949 and 444 aa respectively. Domain architecture of SCD1 protein of *A. coluzzii* (AGAP001713), *D. melanogaster*, *P. falciparum* and human contain central fatty acid desaturase (FA_desaturase) domain, transmembrane domain. SCD1 of mosquito lacks low complexity region, coiled coil region and Cyt-b5 like heme/steroid binding domain. The domains were predicted using the Simple Modular Architecture Research Tool (<http://smart.embl-heidelberg.de>) (Schultz et al. 1998, 2000).

Some other domains (i.e low complexity region, coiled coil region and Cyt-b5 like heme/steroid binding domain) which are present in other orthologues in *D. melanogaster*, *P. falciparum* and human are absent in mosquito SCD1 protein. SCD1 of *A. coluzzii* has 8 paralogues and 49 insect orthologues.

Alignment of the central domain of SCD1 (residues 68 to 309) and the equivalent domain of SCD1 orthologues in humans, *D. melanogaster* and *P. falciparum* was performed using CLUTALW and showed identity rates of 45.13%, 92.21% and 71.88% respectively (Fig. 3.2)

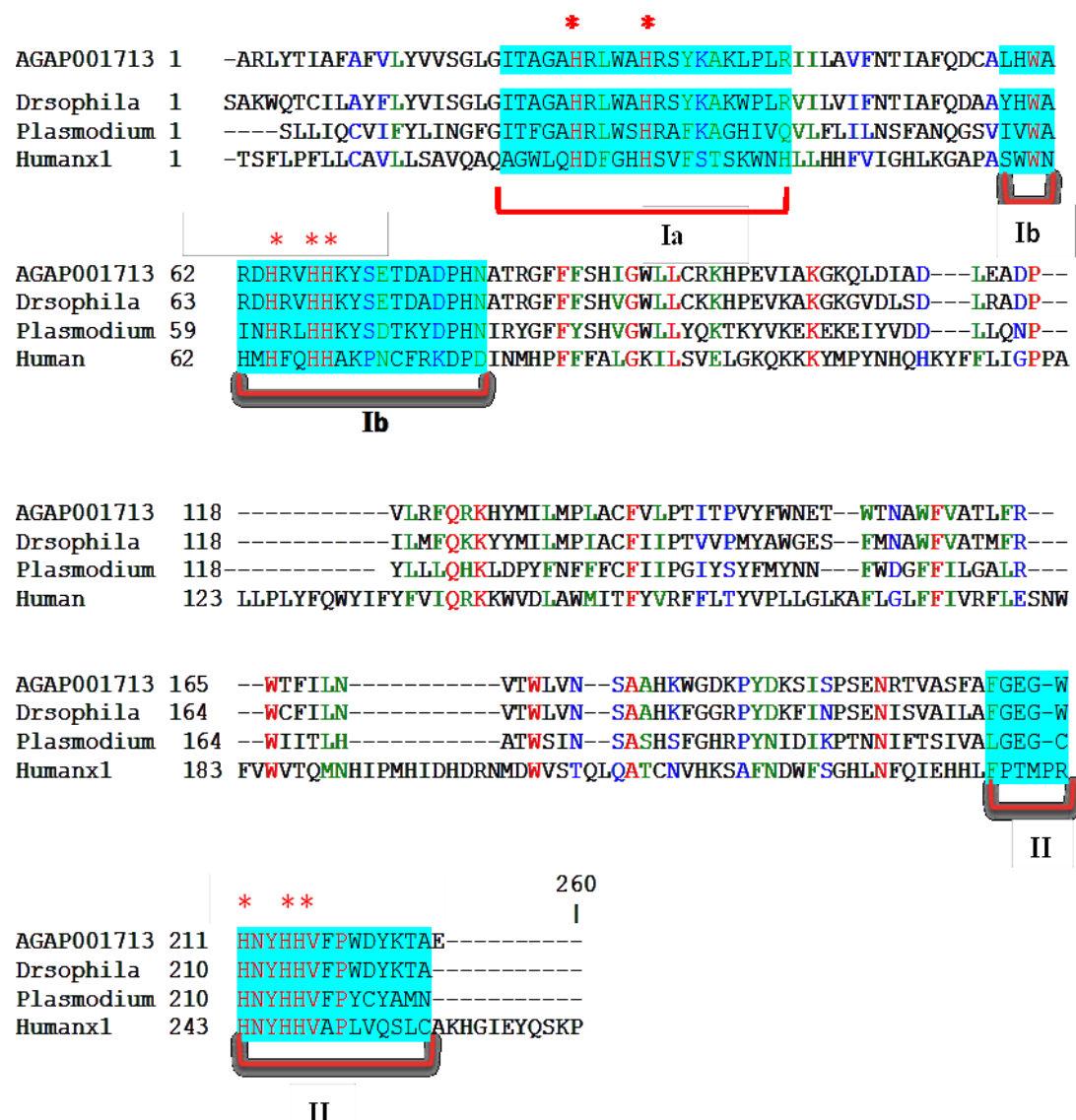


Fig.3.2 Sequence analysis of the *A. coluzzii* Stearoyl- CoA desaturase1

Alignment of the central domain of SCD1 (residues 88 to 303 aa) and the equivalent domain of SCD1 orthologues in humans, *D. melanogaster* and *P. falciparum* was performed using CLUTALW and showed identity rates of 45.13%, 92.21% and 71.88% respectively. The SCD1 domain contains the three Histidine (His) Boxes designated as region Ia, Ib and II and also contain all eight conserved His residues which are essential for Stearoyl-CoA activity.

The *SCD1* domain contains three Histidine (His) Boxes designated as region Ia, Ib and II, which together contain all eight conserved His residues that are essential for Stearoyl-CoA activity (Shanklin et al. 1994). His boxes are important, as electrons and protons bound to cytochrome b5 and released from NADPH by cytochrome b5 reductase coenzyme, attach to the His box through two iron atoms and two electrons to the fatty acyl-CoAs. The protein has one transmembrane domain, predicted using the SMART package.

Silencing *SCD1* is lethal to both blood fed and sugar fed mosquitoes

Age matched (0-2-days old) female mosquitoes were injected with control *LacZ* and *SCD1* dsRNA and kept separately in netted cups and maintained on 5% fructose solution until recovery. *SCD1* silencing levels were assessed by qRT PCR 3 days post recovery (Fig. 3.4C). The knockdown (KD) efficiency was 82%.

For the blood feeding survival experiment, control and ds*SCD1*-injected mosquitoes were fed on human blood through a membrane feeder 3 days post recovery. Fully engorged females were recovered, counted and maintained on 5% sucrose solution throughout the rest of the experiment. Dead mosquitoes from each group were counted every 6 hours post blood meal (PBM). For the sugar feeding experiment, the monitoring of survival began 3 days post injection recovery and continued until all mosquitoes were dead. Throughout the experiment, mosquitoes were maintained on 5% sucrose solution. Dead mosquitoes from each group were counted every 6 hours post blood meal (PBM). For the sugar feeding experiment, the monitoring of survival began 3 days post injection recovery and continued until all mosquitoes were dead. Throughout the experiment, mosquitoes were maintained on 5% sucrose solution.

A dramatic, blood feeding-induced mortality was observed in *SCD1* KD blood-fed mosquitoes within 24-48 h PBM, which reached 100% by 48 h (median survival =42). In contrast, only 17% mortality of blood-fed control mosquitoes was observed at 48 h PBM (Fig. 3.3A). Kaplan–Meier survival statistics showed that these differences in mortality levels between control and *SCD1* KD blood-fed mosquitoes were highly significant *dsSCD1* ($p<0.0001$). Whilst *SCD1* KD mortality rates were evidently slower in the sugar feeding compared to the blood feeding experiment, these rates were highly significant compared to their counterpart sugar-fed controls ($p<0.0001$), which reached 65% at day 6 of post injection recovery (Fig.3.3B). Median survival for *SCD1* KD group was 11.50 and 6.000 for control.

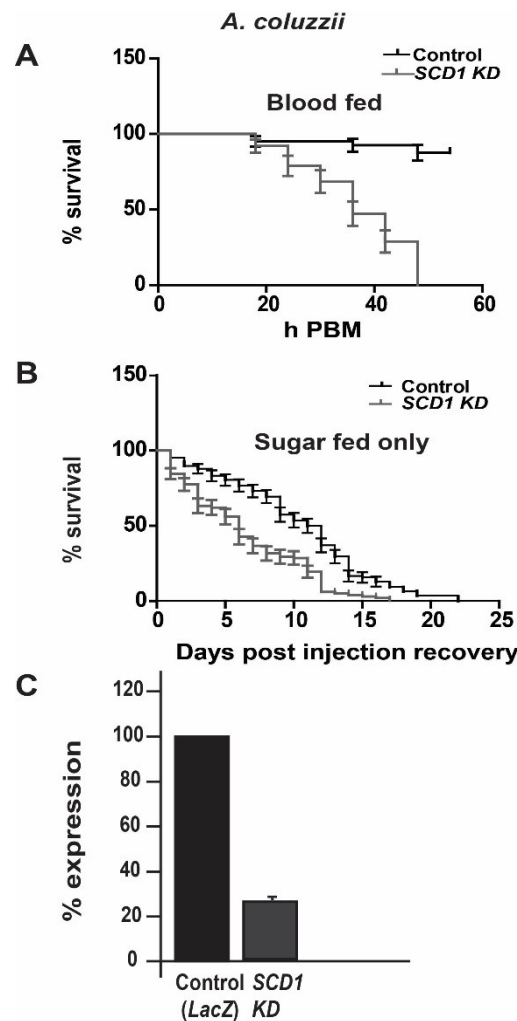


Fig. 3.3 Blood and sugar meal mediated mortality in *SCD1* KD female *A. coluzzii* mosquitoes

(A) Survival analysis of 0-2 day old female mosquitoes injected with *SCD1* or control *LacZ* dsRNA, blood-fed 3 days post injection recovery and monitored every 6 h until all mosquitoes of any one group were dead; all *SCD1* KD mosquitoes died within 48 h PBM. Highly significant differences in mortality rates ($P < 0.0001$) between *SCD1* KD and control mosquitoes were detected by a log-rank (Mantel-Cox test) survival analysis. Median survival for *SCD1* KD mosquitoes was 42. The mean $\pm$ SEM of data obtained from three independent biological experiments is presented. (B) Survival analysis of *SCD1* KD and *LacZ* dsRNA injected (control) female mosquitoes maintained on 5% sucrose for 25 days post injection recovery. Dead mosquitoes were enumerated each day post injection recovery, and the mean $\pm$ SEM of survival data from three independent biological experiments is shown. Log-rank (Mantel-Cox test) survival analysis indicated highly significant differences in mortality rates between *SCD1* KD and control mosquitoes ($P < 0.0001$). Median survival for *SCD1* KD was 11.50 and 6.000 for control. (C) RNAi mediated *SCD1* KD efficiency in female *A. coluzzii* mosquitoes. *SCD1* transcript levels were decreased by 70%.

Silencing *SCD1* causes defects at the organismal, tissue and cellular levels

To investigate the underlying cause of the blood feeding-induced mortality and the overall impact of *SCD1* KD mosquitoes on mosquito physiology, the effects of *SCD1* KD on the midgut structure were analysed. It was hypothesized that *SCD1* KD might have impacted on maintenance of cell membrane fluidity (Natambi, 2002), directly or indirectly inhibiting the mosquitoes ability to cope with the mechanical and metabolic stress of blood feeding.

Light microscopy revealed a remarkable phenotype characterized by blood-filled thoracic cavity of blood-fed *SCD1* KD mosquitoes 24 h PBM, indicating blood perfusion of the hemocoel (Fig. 3.4A). In addition, extremely fragile peritrophic matrix and comparatively bigger and darker midguts were discovered in *SCD1* KD mosquitoes at 24 h PBM (Fig. 3.4B).

To investigate further the effect of *SCD1* KD on the midgut epithelial cell structure, TEM was performed on midgut tissues at 0 and 24 h PBM. The results showed that cell membranes were more distinct and significantly thicker (60 nm) in *SCD1* KD compared to control mosquitoes (31nm) at 0h. Similarly, cell membranes were thicker (55 nm) in *SCD1* KD compare to control (14) at 24h PBM. These thicker cell membranes in *SCD1* KD mosquitoes suggest loss of cell membrane rigidity (Fig. 3.5A, Fig. 3.6).

It was also notable that the cell membrane thickness was not reduced after blood feeding, which is due to the expansion of the epithelial cell surface providing structural support to the engorged midgut epithelium (Sodja, Fujioka, Francisco J A Lemos, et al. 2007). Therefore, it is hypothesized that inhibition of *SCD1* function alters the ability of midgut epithelial cells to undergo the necessary changes needed to offset the increased pressure within the lumen allowing blood to enter into the thoracic cavity by rupturing the epithelial cell layer.

TEM analysis additionally revealed a great deficiency in lipid droplets in midgut cells of *SCD1* KD compared to control mosquitoes at 24 h PBM (Fig.3.8 A bottom panel and 3.8 B). Therefore, blockage of MUFA synthesis catalysed *SCD1* KD inhibits TAG and PL lipid biosynthesis from blood meal-derived carbon in the midgut cells (Amu, 2011).

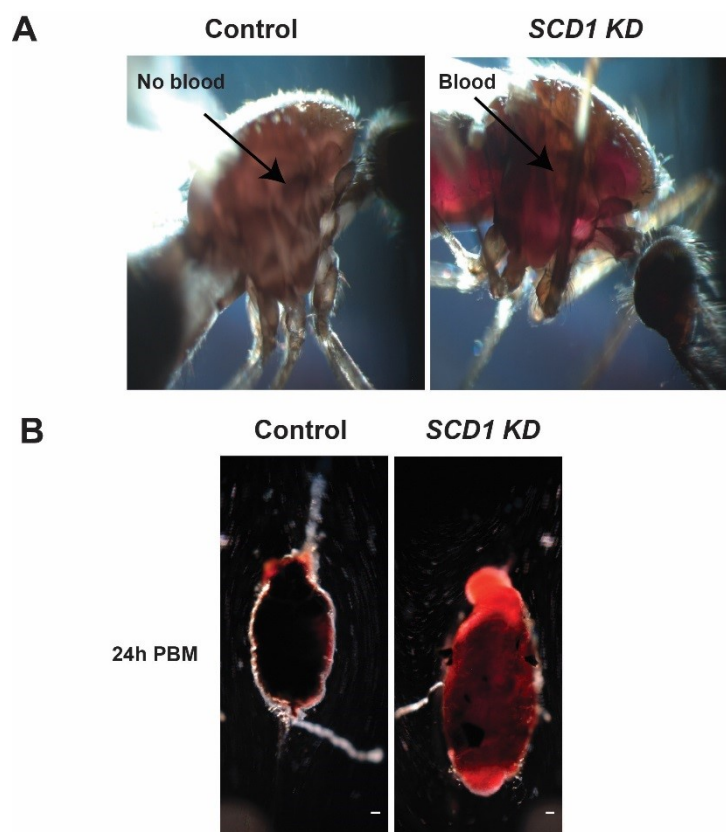


Fig. 3.4 *SCD1* KD causes major physiological and morphological defects in the midguts of female mosquitoes

(A) Representative photos of thoraces of blood-fed control (left) and *SCD1* KD (right) female mosquitoes at 24 h PBM. The red colour of the thorax of *SCD1* KD mosquito indicates blood perfusion of the hemocoel (black arrows). (B) Bigger, darker and fragile midgut/ peritrophic matrix with blood bolus in *SCD1* KD mosquito (Scale bar: 600 μ m).

The disruption of microsomal *SCD1* function increases energy expenditure by increasing the beta-oxidation and electron transport chain in mitochondria. Mitochondria are crucial for cell viability and their morphology and physiology are extremely sensitive to the ratio of SFA/MUFA in the cell (Gomes, 2011). The shape and abundance of mitochondria in the midgut epithelial cells of the *SCD1* KD mosquitoes were examined at 0 and 24 h PBM by TEM. Indeed, fewer, irregularly shaped and indistinct (degenerated) mitochondria were observed in *SCD1* KD cells compared to controls (Fig. 3.7).

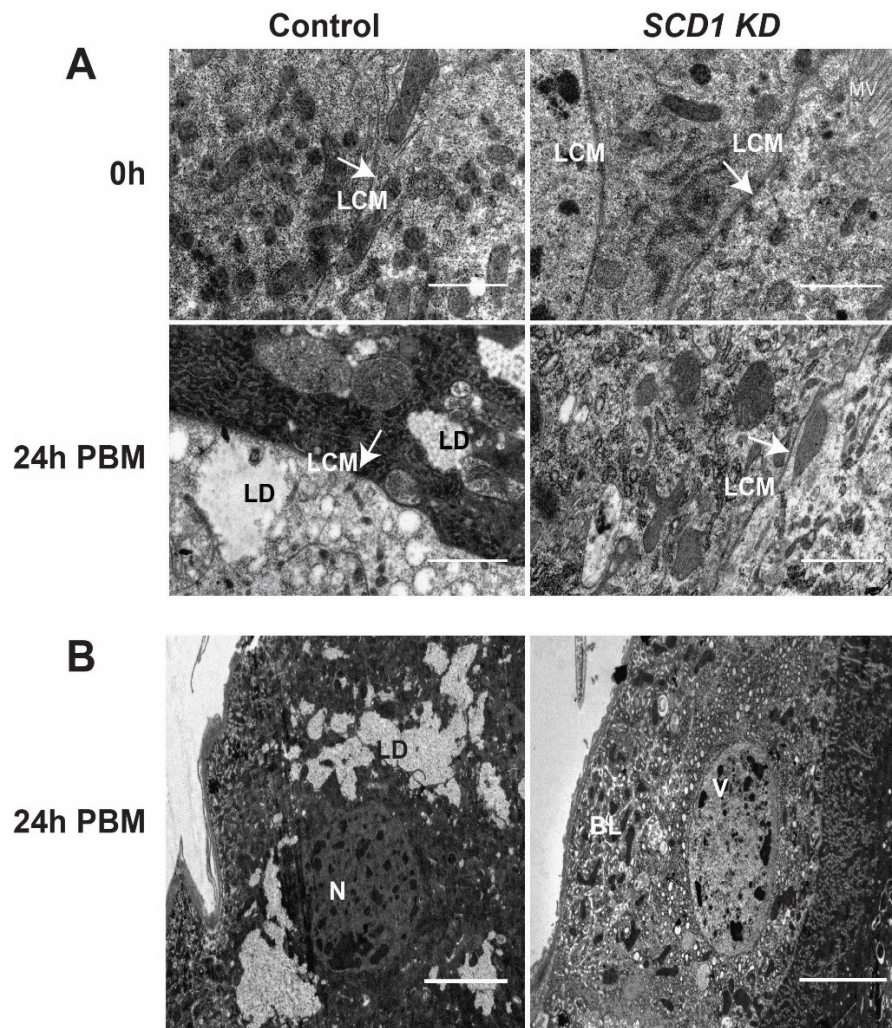


Fig. 3.5 KD of *SCD1* inhibiting MUFA biosynthesis causes midgut cell membrane rigidity and depletion of lipid droplets.

(A) Representative TEM pictures of midgut epithelial cells dissected from control (left) and *SCD1* KD (right) female mosquitoes at 0 h (top) and 24 h (bottom) PBM, respectively. At 0 h, lateral borders between adjacent midgut cells (LCM) are thicker (60 nm) in *SCD1* KD compare to control (31 nm) mosquitoes. LCM is also thicker in *SCD1* KD (55 nm) than in control (14 nm) mosquitoes at 24 h PBM. Thickness of LCM is the mean value of 10 different points on the same LCM. M denotes mitochondria and MV denotes microvilli. Scale bar is set at 1 μ m. (B) Representative TEM pictures of midgut epithelial cells dissected from control and *SCD1* KD mosquitoes at 24 h PBM, highlighting the depletion of lipid droplets (LD). Scale bar is set to 1 μ m. N and BL denote nucleus and basal lamina, respectively.

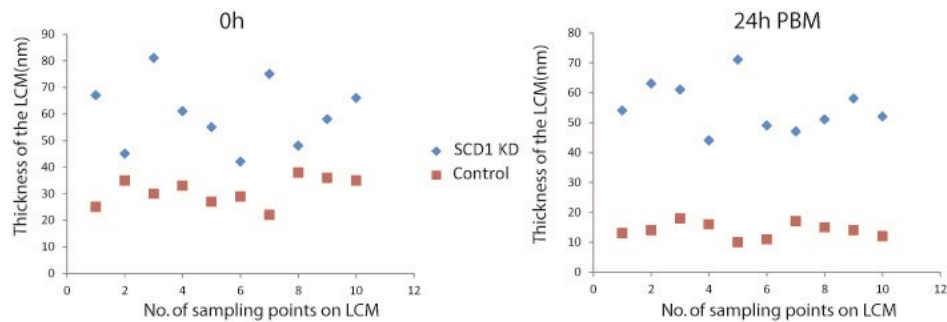


Fig.3.6 Thickness of Lateral cell membranes of midgut cells (LCM) in control and *SCD1* KD mosquitoes at 0h and 24h PBM.

Distribution of values taken from 10 different points on the representative TEM pictures of midgut epithelial cells dissected from control and *SCD1* KD female mosquitoes at 0h (left) and 24h (right) PBM, respectively. Thickness of LCM is the mean value of 10 different points on the same LCM. m

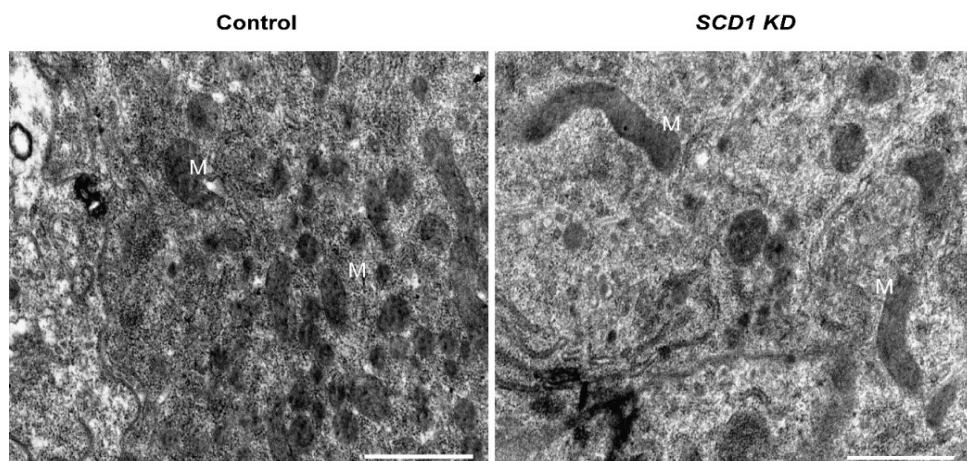


Fig. 3.7 Midgut cells of *SCD1* KD mosquitoes contain fewer, indistinct and irregularly shaped mitochondria.

Representative TEM pictures of mitochondria in control and *SCD1* KD mosquitoes at 0 h PBM. The scale bar sets to 1 μ m.

This particular finding contradicts with previous studies where scientist found that insects increase volume and activity of mitochondria to provide more energy to the body during the

flight. However, the decreased number of mitochondria could be the result of cell depolarization due to the increased level of SFAs (Zheng, 2008; Derek, 2008).

It was also observed that blood-fed female *SCD1 KD* mosquitoes do not show signs of egg development. The ovary is a major target tissue of Lp-carried lipids synthesized by the conversion of blood meal-derived carbon to TAG and PL (substrates for cholesteryl esters), the major components of the mosquito Lp (Amu, 2011), and *SCD1* is required for the biosynthesis of MUFA (Natambi, 1999). The cellular and sub-cellular morphology of ovaries were therefore examined by light and TEM microscopy. Lp inhibition has major effects on ovary development (Atella et al. 2006, Vlachou et al. 2005). Indeed, ovaries of *SCD1 KD* mosquitoes remained immature and significantly smaller in size compared to control mosquitoes at 24h PBM (Fig. 3.8). Therefore, it hypothesised that insufficient synthesis of lipid droplets in the midgut cells due to the inhibition of *SCD1* activity, the major component of developing eggs (Ziegler, 2006), is responsible for reduced lipid shuttling in the hemolymph leading to undeveloped ovaries.

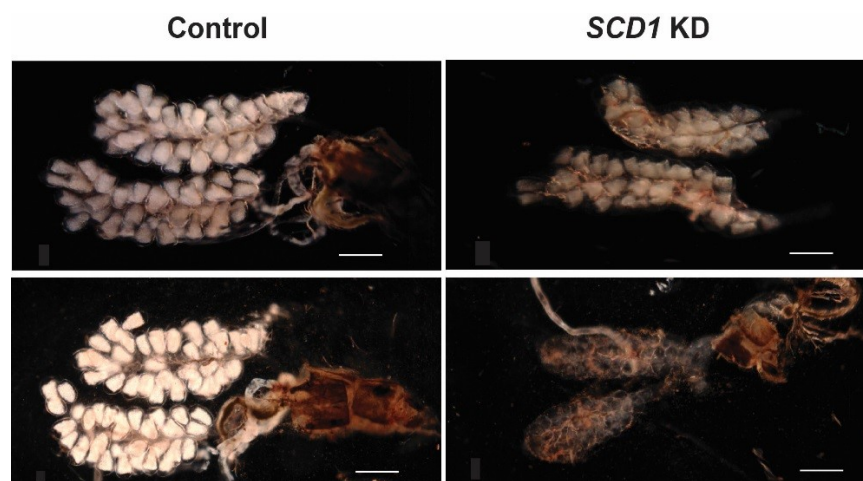


Fig. 3.8 Silencing *SCD1* leads to undeveloped ovaries in blood fed mosquitoes.

Sets of light microscopy pictures of representative ovaries dissected from control (left) and *SCD1 KD* (right) female mosquitoes at 24 h PBM. *SCD1 KD* causes undeveloped ovaries compare to control mosquitos. Scale bar is set to 600 μm .

Discussion

Phospholipid and neutral lipid biosynthesis are key to many aspects of prokaryotic and eukaryotic cell biology such as cell growth, proliferation and signalling (Baenke, Peck, Miess,

& Schulze, 2013; Ntambi, 1999). Deregulation of lipid metabolism can lead to various metabolic syndromes and physiological abnormalities in mammals and insects (Ntambi & Miyazaki 2004; Alabaster et al. 2011). The results presented here reveal that fatty acid $\Delta 9$ -desaturase activity, encoded by *SCD1* and catalysing the synthesis of MUFA from SFA, is essential for survival, blood meal digestion and reproduction in adult female *A. coluzzii* mosquitoes. Loss-of-function of *SCD1* by RNAi-mediated gene silencing leads to female mosquito mortality as early as 48 h after a blood meal although significantly reduced survival rates are also observed during a sugar meal.

SCD1 is a short-lived, polytopic, membrane-bound, non-heme and iron-containing enzyme, primarily localized in the endoplasmic reticulum. It synthesizes MUFA, a key fuel source, from SFAs by introducing a double bond into acyl-CoAs between the 9th and 10th carbon molecules (Miyazaki et al. 2003; Heinemann & Ozols 2003). This is a critical process, as overloading with SFAs can induce a cell toxic response known as “lipotoxicity” which causes a variety of diseases in humans including hepatic disease, type-2 diabetes, insulin resistance, atherosclerosis and coronary heart disease (Park et al. 2014). In particular, it is shown that excessive accumulation of palmitic acid, the most common SFA, induces apoptotic cell death by inducing ER stress (Zhang et al. 2012; Gu et al. 2010; Park et al. 2014), whereas inhibition of SCD1 activity causes impaired metabolism-induced programmed cell death in cancer cell lines (Miyazaki et al. 2007). Finally, loss of *SCD1* activity leads to significant excess accumulation of SFAs and reduced viability of different stages of *Plasmodium* parasites, yeast and chick embryos (Gratraud et al., 2009, Austic, Hill, & Wilson, 1971, Cook & McMaster 2002).

The dramatically induced mortality of *SCD1* KD mosquitoes, especially after a blood meal, is thought to be the combined result of toxic accumulation of SFAs and depletion of MUFAs. The phenotype consists of significant blood meal-leakage into the mosquito body cavity in conjunction with thicker lateral cell membranes (LCM) of midgut cells suggesting that the midgut epithelium integrity is compromised as a result of *SCD1* silencing. Since several signalling molecules are derived from fatty acids or intermediate metabolites in the fatty acid synthesis pathway like cholesterol, a bigger and darker midgut, associated with undigested blood bolus, could be the result of an interrupted feedback signalling mechanism, initiated in the fat body which serves to coordinate blood meal digestion in multiple tissues (Alabaster et al. 2011).

As described previously, SCD1 introduces double bonds into saturated fatty acids and maintains an optimum ratio of SFAs to MUFAs, a homeostatic function that is vital for membrane fluidity and curvature of the cell (Ntambi, 2004). Double bonds cause bends in

fatty acyl chain and decrease rigidity, markedly influencing the fluidity, permeability and stability of biological membranes (Cook & McMaster 2002). Therefore, increased levels of SFAs cause thicker, stiffer and stickier cell membranes (Moritz, 2014). Cytological studies of *An. gambiae* mosquitoes showed that, after blood feeding, the membranes of midgut epithelial cells are reduced in thickness by 80% as a result of the dramatic gut expansion to accommodate the blood meal (Sodja, Fujioka, Francisco J A Lemos, et al. 2007). Taken together, these results suggest the lack of optimal feedback processes for blood meal digestion following *SCD1* KD leads to breakdown of the homeostatic function necessary for maintaining the structural integrity of the mosquito midgut epithelium. As a result, the un-metabolized blood creates pressure on the epithelial cell layer, which ruptures, releasing blood into the thorax. Furthermore, accumulation of SFAs is likely to cause ER stress-like induced morphological changes that additionally alter the ability of the midgut to expand after a blood meal. The latter is supported by a previous study reporting a similar phenotype for the coatamer protein (COPI), an essential component for protein secretion and lipid droplet formation. Silencing *COPI* in *Ae. aegypti* mosquitoes causes ER stress- like reorganisation and reassembly contributing to a weakening of the cell layer of the midgut epithelium (Isoe et al. 2011).

The functionality of membranous organelles such as mitochondria and ER depends on the biophysical properties of their biological membranes. Manipulation of FA saturation can dramatically change these properties and affect many features of the cellular machinery. *SCD1* inhibition increases β -oxidation by enhancing expression of palmitoyltransferase 1 which transport fatty acids to mitochondria. Flight muscle cells which involved in regular and persistent physical activity of insects like locust can increase their mitochondrial activity and mass to accomplish their goal (Saql, 2000). However, mitochondrial biogenesis is tissue specific in higher organisms in response to exogenous and endogenous regulators. Moreover, MUFA upregulates mitochondria biogenesis and induces β -oxidation in white fat cells (Flach, 2005) and high fat diet or saturated fatty acid exposure downregulates mitochondrial biogenesis in skeletal muscle (Sparks,2005). Dilated and a decreased number of mitochondria in the midgut cells of *SCD1* KD mosquitoes at 0h and 24h PBM was observed. Increased levels of SFAs degrade mitochondria, which is associated with depolarization in cancer cells (Li et al. 2008; Narendra et al. 2008). Consistent with this idea, Recently, it has been reported that mitochondria were found to be dilated after palmitic acid exposure, which lead to reduced cell viability in human Chang Liver Cells (Park et al. 2014).

Ingestion of a blood meal by female mosquitoes triggers a number of dramatic morphological and physiological changes through a network of metabolic pathways. In particular, blood meal nutrients are converted into lipids that are stored in the fat body in the form of lipid

droplets by the loading and unloading activities of Lp from the midgut to the storage site (fat body), thus providing metabolic energy and maternal and egg lipids (Amy 2011, Isole, 2011, sojda2007, GUOLI, 2009, Amy, 2011). In anautogenous mosquitoes, oocyte development involves the rapid accumulation of a large amount of fat, especially TAG, following blood meal; an oocyte contains 30-40% lipid. Since inhibition of *SCD1* hinders the biosynthesis of TAG and cholesterol, which are chief components of lipid droplets, it is concluded that loss of *SCD1* compromises the development of ovaries and oocytes due to an insufficient amount of lipid droplets.

Similar phenotypes, i.e. an undeveloped ovaries and undigested blood bolus, have been reported in a study where deficiencies of the key rate-limiting lipid biosynthetic enzyme acetyl-CoA carboxylase (ACC) produced defective oocytes, which lacked an intact eggshell and gave rise to unviable eggs and delayed blood meal metabolism in *Ae. aegypti* (Alabaster et al. 2011)

Chapter 4

Gene expression and metabolic phenotypes in *SCD1* KD *A. coluzzii*

Summary

The investigation of the associations between genome-wide expression, metabolic profile and phenotype is a powerful approach for the understanding of a biological process. Genome-wide transcriptional and metabolomic profiling analyses were carried out in order to provide insights into the dramatic phenotype of adult *Anopheles coluzzii* mosquitoes following silencing of the Stearoyl CoA desaturase (*SCD1*) gene reported in Chapter 3 of this thesis. Transcriptional profiling using oligonucleotide DNA microarrays revealed a large number of genes (601) that were differentially expressed before and/or after a blood meal in *SCD1* KD mosquitoes. Most of the downregulated genes were functionally related to carbohydrate, lipid or protein metabolism, while replication, protein synthesis and oxidative stress were also affected. The majority of genes related to immune response, signalling and cytoskeleton organization and biogenesis were markedly upregulated. Metabolomic profiling showed that *SCD1* KD caused a significant reduction of standard fatty acid desaturase indices, indicating an overall increase of saturated fatty acids and a parallel decrease of unsaturated fatty acids in the mosquito body. A large number of additional metabolites were identified to be differentially regulated in *SCD1* KD compared to control mosquitoes. In response to blood feeding, the levels of several amino acids and many substrates of the TCA cycle increased while intermediates of glycolysis and the pentose phosphate pathway decreased. These data highlighted the biochemical framework by which the *SCD1* KD phenotype is developed after a blood meal leading to a major metabolic syndrome. They additionally indicated that these metabolic conditions might be triggering an auto-inflammatory response evidenced by the upregulation of immune responses.

Introduction

Lipids play pivotal roles in a number of important processes linked to cellular and tissue maintenance such as cellular membrane biosynthesis, signal transduction, energy storage, assembly of lipoprotein particles, protein modification and many more. In order to maintain normal cellular functions, intracellular levels of lipids are tightly regulated by a network of metabolic pathways. Disturbance of the cellular SFA to MUFA profile through inhibition of the endoplasmic reticulum-anchored *SCD1* enzyme may yield diverse genetic, metabolic and systemic effects, including stress and cellular inflammatory responses (Minville-Walz et al. 2010). For example, chronic inflammation has been recorded in the pathology of obesity, which is closely related to overexpression of *SCD1*.

Phenotypic characterization of *SCD1* gene in *A. coluzzii*:

Fatty acid $\Delta 9$ -desaturase activity is required for the conversion of saturated (SFAs) to mono-unsaturated (MUFAs) fatty acids that serve as precursors of major structural components of the cell membranes and are essential for survival, reproduction and blood meal digestion in adult female *Anopheles* mosquitoes. Here, the *A. coluzzii* Stearoyl-CoA desaturase 1 (*SCD1*), encoded by the VectorBase gene AGAP001713, is phenotypically characterized through a series of experiments that involved RNAi-mediated gene silencing in adult female mosquitoes. The mortality rate of *SCD1* KD mosquitoes was decreased during sugar feeding and dropped dramatically immediately after a blood meal; all mosquitoes died by 48 h post blood meal. Microscopic observations revealed that the body cavity of dead *SCD1* KD mosquitoes was filled with blood suggesting that the midgut epithelial integrity was severely compromised. Transmission electron microscopy showed that the midgut cell membranes are thick and rigid, and fail to extend and thin out after a blood meal. This is consistent with the homeostatic function of *SCD1* in maintaining an optimum ratio of SFAs to MUFAs, which is vital for cell membrane fluidity and plasticity. In addition, midgut cells of *SCD1* KD mosquitoes contained fewer, indistinct and irregularly shaped mitochondria, presumably due to altered energy metabolism and cell depolarisation; it is known that mitochondrial morphology and physiology are sensitive to the SFA/MUFA ratio. Finally, midgut cells of *SCD1* KD mosquitoes showed a substantial depletion of lipid droplets after a blood meal, the main energy and lipid storage source, while ovaries did not develop and remained small in size.

Relationship between TOR signalling pathway and *SCD1*:

The target of rapamycin (TOR or mTOR for mammalian TOR) is a candidate pathway for being involved in the regulation of lipid metabolism. TOR is a serine/threonine protein kinase

protein belonging to the phosphatidylinositol 3-kinase (PI3K) superfamily. It is a central regulator of the nutrient sensing signal transduction pathway which is conserved from yeast to mammals (Rohde et al. 2001). TOR controls cell growth, proliferation, motility and survival, gene expression and protein biosynthesis via transcriptional and translational regulatory pathways. The fat body of mosquito secretes yolk protein precursor (YPP) into the hemolymph that is uptaken by developing oocytes via receptor mediated endocytosis. Several signalling pathways regulate vitellogenic YPP synthesis (Hansen et al. 2004). Mosquitoes regulate *YPP* gene expression via the TOR signaling pathway that conducts the amino acid mediated signal. After a blood meal, mosquitoes produce free amino acids largely through the metabolism of hemoglobin and proteins. It has been found that interruption of TOR signalling diminishes *YPP* gene expressions and egg production (Attardo et al. 2006; Carpenter et al. 2012). Downstream targets of TOR signalling, such as S6 kinase and translation initiation factor binding protein (4E-BP), play crucial roles in egg development in *Ae. aegypti* (Hay 2004; Hansen et al. 2005). The relationship between TOR signalling and *SCD1* has been established in mammalian breast cancer cells. It has been shown that rapamycin (mTOR inhibitor) and its analogues decrease *SCD1* expression by inhibiting *SCD1* translation initiation factor binding protein (4EBP1) and Sterol regulatory element binding protein1 (SREBP1), perhaps through the mTOR/eIF4E-binding protein 1 axis (David Luyimbazi et al. 2010).

Inflammation and SFAs:

Current studies recognize SFAs, especially free fatty acids FFA, as active proinflammatory factors in a variety of cell types including macrophages, hepatocytes and myocytes. During the process of inflammatory signalling, SFAs serve as ligands to immune receptors such as the inflammasome, insulin pathway and Toll- like Receptors (TLR) pathways. This leads to activation of mitogen-activated protein kinase 1 and NF- κ B that transcribe downstream effectors involved in potent pro-inflammatory responses. Other lipid intermediates, such as DAGs and ceramides, also act as proinflammatory factors. Prolonged and unchanging inflammatory signals, caused by altered levels of bioactive lipids initiate cellular stress responses and dysfunction, consequently resulting in cell death and systemic degeneration in a process called lipotoxicity (Liu et al. 2011). However, till to date it is unknown if SFAs have proinflammatory function in insect system.

DNA microarray: A tool for analysis of gene expression

DNA microarray, a revolutionary tool for global analysis of genome sequences and gene expression, is a collection of a group of DNA spots attached to a solid surface. Each sample DNA spot typically contains picomoles of specific DNA sequence known as probe (Trevino &

Falciani 2007). There are mainly two types of DNA arrays depending on the spotted samples (probes): one array uses small single stranded oligonucleotides which are ~22 nt long. The other type of arrays uses complementary DNA (cDNA) or their open reading frames (ORF). These probes bind to the complementary unknown sequences hence allow parallel analysis for gene expression and gene discovery. Among these two arrays oligo-based DNA arrays is more specific for the target gene (Trevino & Falciani 2007).

The most common and basic uses of DNA microarray is to analyse specific molecular differences in the genes that are associated with a specific biological effect. These analyses can be used to develop new hypotheses and guide the design of further experiments (Trevino & Falciani 2007). This high-throughput expression profiling tool can be used to compare the levels of gene transcription in specific conditions, such as gene KD or drug treatments in order to monitor the variation in gene expression in response to special conditions, to understand the mechanisms involved in metabolic syndrome development or to identify diagnostic biomarkers (Tarca et al. 2006). Microarray data analysis also helps to detect co-expressed genes. Finding genes with similar expression profile therefore may reveal potential clinical targets, genes with similar biological functions, or expose novel biological connections between genes (Getz et al. 2000). The identification of these single nucleotide polymorphisms (SNPs) is an important tool for identifying genetic loci linked to complex disorders (Piotrowski et al. 2006). DNA microarrays technology can also be used to detect the presence of hundreds of pathogens in a single experiment. Specific sequences from pathogens can be spotted on a microarray to build a purpose-specific microarray. Then the extracted genomic DNA from a patient tissue, or from a food sample suspected to be infected, is hybridized to the microarray (Wang et al. 2002; Conejero-Goldberg et al. 2005).

Metabolome analysis : A modern tool for metabolite profiling

Metabolites refers to the intermediates and products of metabolism (small molecules with molecular weight of <1000 Da in natural product research. Metabolites which are essential for the survival, growth and reproduction of organism via normal metabolic processes known as primary metabolites such as amino acids, lipids, and carbohydrates. Compounds those are non-essential but necessary for survival in a given environment known as secondary metabolites i.e. polyphenols, alkaloids, terpenes and hormones (Wolfender et al. 2015).

Metabolite analyses on a sufficient number of replicates help researchers to divide samples into different groups and monitor changes in metabolome composition in a given situation like physiological status, a stress or stimulus, genetic alteration or contact with other organisms. Several analytic strategies are used to detect the chemical composition of a particular biological matrix or extract. Among different analytic strategies metabolite

fingerprinting, metabolite profiling, and metabolite target analysis are more often used in bio-research. Metabolite fingerprinting is used to determine differences and classify samples instead of metabolite identification and quantification. Metabolite profiling is the oldest, well accepted analysis and more targeted than metabolite fingerprinting. This high-throughput analysis focuses on a large group of metabolites that is either related to a specific metabolic pathway or a class of compounds (Wolfender et al. 2009). Therefore, this rapidly evolving tool in systems biology is metabolomics that can be used to identify metabolites in biofluids, cells, tissues, and organisms and to observe the metabolic flux which follows genetic modification, pathophysiological changes, or exogenous challenges (Chen et al. 2008).

Traditionally, gas-chromatography mass-spectrometry has been used as technological platform for metabolite profiling to detect common or expected metabolites in both plant and non-plant species (Plumb et al. 2003). For rapid screening for common metabolites a list of potential metabolite masses have been developed by using ion scanning techniques that use rule-based algorithms. Single LC/MS analysis can be used to detect both expected metabolites and the acquisition of their product ion spectra (Gangl et al. 2002; Lafaye et al. 2004). This analysis is highly successful in screening predicted metabolites using comprehensive metabolite databases developed from knowledge of biotransformations (Xia et al. 2003). The robustness of the metabolite prediction algorithms becomes a critical factor since these analyses can only detect predictable metabolites that are listed in the database.

The aim of the experiments described in this chapter was to investigate the underlying effects of *SCD1* inhibition on gene expression and cellular metabolites through the use of DNA microarrays and metabolomics.

Methods

Gene silencing

Silencing of *SCD1* through injection of dsRNA in freshly emerged female mosquitoes is described in Chapter 3.

Mosquito colony maintenance

Maintenance of *A. coluzzii* mosquitoes of the Ngousso colony is described in Chapter 3.

Gene expression profiling

Control (*dsLacZ* injected) and *SCD1* KD female mosquitoes were fed on human blood through a membrane feeder 3 days after recovery, and 10 fully-engorged female mosquitoes

from both groups were sampled at each of the following time points: 0 (just prior to BF), 6, 12, 18 and 24 h PBM.

Total RNA was extracted using the TRIzol reagent (Invitrogen) and purified using the RNeasy kit (Qiagen). RNA quantification was performed using the Nanodrop 1000 spectrophotometer (Thermo Scientific) and RNA quality and integrity was assessed through gel electrophoresis (1% agarose gel) and the Agilent Bioanalyzer 2100 (Agilent Technologies). Labelling and hybridization were performed, following the instructions in the Low Input Quick Amp Labeling kit, for two-colour microarray-based expression analysis (Agilent). The Pfalcip_Agamb2009 microarray platform (designed by Dr. Vlachou, personal communication) was used for all of the hybridizations presented. This platform encompasses a total of 15,424 *A. gambiae* (and *A. coluzzii* alike) annotated transcripts of the AgamP3.8 according to the 2014 VectorBase release along with *P. falciparum* probes. *A. coluzzii* genome assembly suggests 14,712 gene transcripts and shows minor differences from *A. gambiae* transcription profile. Slides were scanned using the Genepix 4000B scanner equipped with Genepix Pro 6.1 software (Axon instruments).

All dataset files were normalized using Genespring 11.0 GX software (Agilent). The Lowess Normalization Method, with the threshold of raw signals set to 50%, was used. Significant differences in gene expression between experiment and control groups were evaluated by combining an expression ratio cut-off of 1.0 on a log₂ scale and one-way ANOVA statistical tests (with P values of ≤ 0.025) across different time points. Expression profiles were clustered using Cluster software, version 3.0, according to the Pearson correlation score. The data were visualized with Java TreeView version 1.1.6r4. All subsequent data analysis was done in Microsoft Excel (Microsoft Corp., Redmond, WA). Quantitative real-time RT-PCR (qPCR) was performed as described in Chapter 3 to validate gene expression results obtained from microarray analysis.

Metabolomic Profiling

For the *SCD1* KD profiling, newly emerged (0-2 days old) female mosquitoes were injected with control *LacZ* or *SCD1* dsRNA and maintained on 5% sucrose solution. They were then fed on human blood through a membrane feeder 3 days later. Fully-fed female mosquitoes from both the control and experimental groups were sampled at 0 (just prior to blood feeding), 18 and 36 h PBM.

For the *SCD1* drug assay, 4 day-old female mosquitoes were offered a human blood meal, supplemented either with 1mM Sterculic acid or 20 mM Tris buffer (control), and maintained

on 5% sucrose solution. Sampling of mosquitoes was performed at 0 (just prior to blood feeding), 18 and 36 h PBM.

Sample preparation and analysis was performed as previously described (Fuchs et al. 2014). In brief, 3 mosquitoes, from each time point, were extracted in a 1 ml ice-cold solution of methanol:water (8:2 v/v) and centrifuged (14000 rpm, 4^o C, 15 min) to separate the cell debris from the supernatant. The supernatant was then transferred to a silanized 1.5 ml glass vial (Agilent Technologies UK Ltd) and dried in a SpeedVac concentrator (Eppendorf). Methoxymation, in combination with trimethylsilylation (methoxy-TMS), was accomplished for derivatization. Sample analysis was performed on an Agilent 7890 GC coupled to a 5975c mass spectrometer, using the Fiehnlib settings. Myristic-d27 acid was used as the endogenous reference. Deconvolution and integration of the extracted metabolites was performed using a coupled AMDIS-Gavin approach. All experiments were carried out in 5 independent biological replicates with 3 technical replicates per each biological replicate. To produce heat maps, metabolite data was normalised to the internal standard (Myristic-d27 acid) and then the averages of each metabolite from five replicates for control or treated group at different time points were calculated. Finally, each metabolite was calibrated to the highest value for that metabolite across all time points and groups. Matlab was used to plot the data.

Pathway Analysis

Pathway analysis was based on the Kyoto Encyclopaedia of Genes and Genomes (KEGG) using transcriptomic and metabolomic data.

Antibiotic treatment and quantitative real-time RT PCR

To reduce the natural microbiota load in the midgut, mosquitoes were treated with antibiotic solutions as described in (Stathopoulos et al. 2014). In brief, mosquitoes were collected just after emergence and kept on a cocktail of 25 µg/ml gentamicin, 10 µg/ml penicillin and 10 units/ml streptomycin, diluted in 5% D-(-)-Fructose (Sigma). This antibiotic treatment continued for 5 days, with the antibiotic solution refreshed every 24 hours. Total RNA was extracted from 10 *dsSCD1* and *dsLacZ*-treated mosquitoes per replicate. cDNA synthesis from total RNA was performed following the manufacturer's instruction in the QuantiTect Reverse Transcription kit (Qiagen). Gene silencing efficiency was determined using the SYBR Premix Ex Taq kit (Takara), according to the manufacturer's instructions, and the ABI Prism 7500 Fast Real-Time thermocycler (Applied Biosystems). The *Anopheles* ribosomal gene *S7* was used as the endogenous reference, and *CEC1* gene expression was quantitated relative to a calibrator control sample (i.e. *dsLacZ*-injected mosquitoes). qRT

primers for *SCD1* gene are described in Chapter 3 and primers for S7 have been reported previously (Habtewold, 2008). Primers for the bacterial 16s RNA have been reported previously (Stathopoulos et al. 2014)

Results

SCD1* KD significantly alters gene expression in female *A. coluzzii

In order to investigate the molecular mechanism underlying the phenotype of *SCD1* KD female mosquitoes in response to blood feeding, a time course of gene expression profiling of *SCD1* KD versus control *dsLacZ* injected mosquitoes was performed. Five time points were examined including 0 (just prior to blood feeding), 6, 12, 18 and 24 h post feeding (PBM) on human blood through a membrane feeder, respectively. Labelled RNA samples of each of *SCD1* KD time point were hybridized against their respective control using two-colour hybridizations of oligonucleotide DNA microarrays. Three independent biological replicates were performed and the data obtained were normalized using GeneSpring.

After normalization, 1432 genes registered expression values in at least one of the 5 time points. Genes with at least 2-fold expression changes in *SCD1* KD mosquitoes versus control mosquitoes in each time point were processed for further analysis ($p < 0.05$ in ANOVA t-test). These differentially regulated genes per time point were functionally classified as per Mendes et al. (2011), into 9 major categories to provide a general picture of changes in cellular gene expression over time.

Overall, gene upregulation dominates downregulation in most of the time points (0, 6, 18 and 24 h PBM; Fig. 1). At 12 h PBM the number of downregulated genes (44) outweigh the upregulated genes (39). The least differential regulation activity was observed at 0 h PBM (75 genes), while the highest differential regulation activity was observed at 24 h PBM (202). At 6 h PBM, the number of regulated genes was almost double that of 0 h PBM. The increase in the number of differentially regulated genes may be explained by the finding that a blood meal triggers a complex and dynamic program of gene expression which includes the activation and/or upregulation of genes required for physiological, morphological and hormonal changes (Sodja et al. 2007).

Immune, signalling and cytoskeleton responses in *SCD1* KD mosquitoes

A large number of genes related to immune response, signalling and cytoskeleton reorganization and biogenesis were markedly upregulated across all time points (Fig. 4.1). Genes belonging to the 'immunity' category became increasingly and proportionally more

numerous with time progression within the *SCD1* KD upregulated gene set. Across the 5 time points, a total of 86 immune related genes were significantly differentially regulated; of those, only 12.7% were downregulated. Immune-related genes represented 15.5 % of all upregulated genes with meaningful annotations at 0 h, reaching 48% at 18 h PBM, indicating a strong immune response as a consequence of blood feeding.

Cytoskeleton/cell adhesion/structural components:

27 genes of this category showed differential expression profiles throughout the time course experiment; 62.9% of them were upregulated (Fig. 4.1 and Table S1). Importantly, upregulation of an actin-like gene was detected at 6 h PBM. It has been reported previously, that increased levels actin mRNA at 3-4 h PBM correlates with a remodelling of epithelial cells for the dramatic distention of the midgut after blood ingestion (Sodja, Fujioka, Francisco J.A. Lemos, et al. 2007). As *SCD1* inhibition causes stiffness of the epithelial cell membranes (see Chapter 3 and Ntambi 1999), it is suggestive that this upregulation represents a feedback response to overcome the mechanical pressures produced by rigid cell membranes in *SCD1* KD mosquitoes due to skewed SFA/MUFA ratios.

E- Cadherin, belonging to a group of calcium-dependent cell adhesion proteins involved in cell-cell interactions is over-expressed in *SCD1* silenced breast cancer cells (Mauvoisin et al. 2013). Consistent with this finding, an upregulation of gene encoding a cadherin-like protein was observed at 24 h PBM. This protein is previously shown to be expressed in the midgut of *A. gambiae* larvae, where it plays a key role in detoxification of nonchemical larvicide-Bti (Hua et al. 2008). Therefore, it is possible that the overexpression of this protein in *SCD1* KD mosquitoes serves in detoxifying excess SFAs.

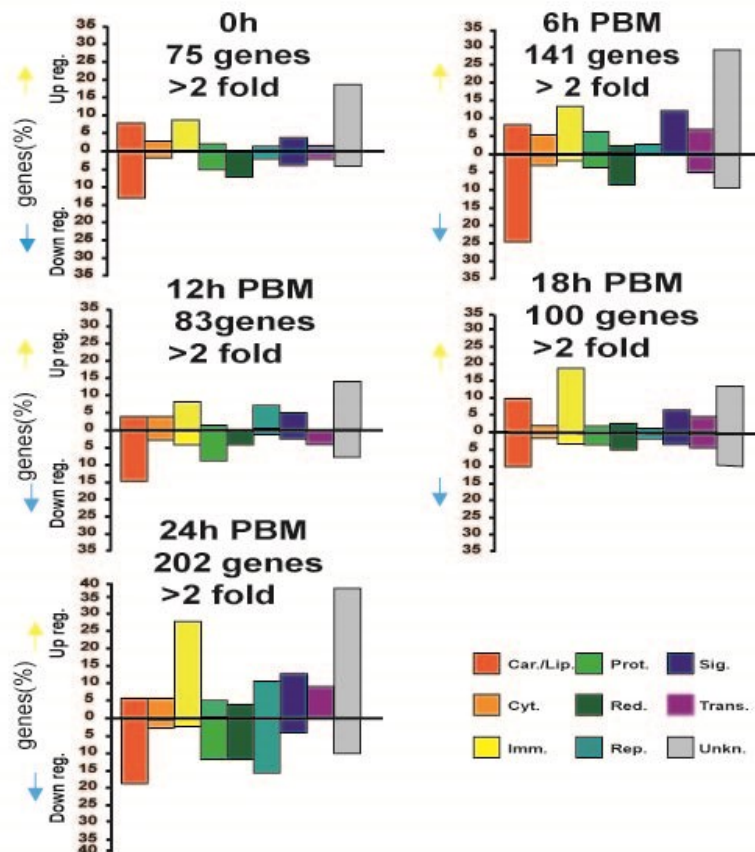


Fig. 4.1 Time course analysis of differentially functional gene categories in *SCD1* KD mosquitoes

Data presented here depicts the fractions of up- and downregulated genes, belonging to distinct functional classes. Car./Lip., carbohydrate and lipid metabolism; Cyt., Cytoskeleton/cell adhesion/structural components; Imm., immunity/putative immunity/phagocytosis; Prot., Protein degradation/proteasome; Red., Redox/ apoptosis/detoxification; Rep., Replication/transcription/translation/transcription factors/ cell cycle; Sig., Signaling/ATPase/GTPases; Trans., Transport /vesicle mediated transport; Unkn., Unknown. Only those genes were considered for this analysis for each time point which exhibited at least 2-fold expression changes (on a log2 scale). Among the 5 time points, the highest numbers of genes were regulated at 24h PBM followed by 6h PBM. Genes belonging to the Carb/Lip., Prot., Rep., and Red. categories were mostly downregulated while genes related to the Imm., Rep., Sig., and Cyt. categories were mostly upregulated.

A gene encoding a chitin-binding domain protein found particularly in the peritrophic matrix (PM) was consistently downregulated throughout the time course (but 18 h PBM), with a peak >5-fold decrease at 24 h PBM. It has been shown that in *A. gambiae*, PM is detectable as early as 12h PBM by EM and fully synthesized by 48 h PBM (Dana et al. 2005). Therefore, it is hypothesized that PM synthesis may be compromised in *SCD1* KD mosquitoes owing to the downregulation of this gene.

Signalling/ATPase/GTPase:

52 genes belonging to this category were regulated throughout the time course (Fig. 4.1). 76.9% of genes in this category were upregulated, mostly at 6 h PBM (27.5% of all upregulated genes at this time point) and 24 h PBM (35% of all upregulated genes at this time point). The gene encoding subunit alpha of the Na/K-transporting ATPase, which catalyses the cation transfer across the membrane by converting ATP to ADP, was upregulated at 6, 12 and 24 h PBM between 2 and 4 fold.

Other signalling genes encoding an odorant binding protein and juvenile hormone binding protein (JHBP) were also upregulated at 24 h PBM. Juvenile hormone (JH) plays important roles in larval development, adult reproduction and stress management. It also suppresses the rate of PM synthesis and function in adult *Calliphora erythrocephala* (Zhu et al. 2003). JH levels drop when JHBP levels increase (Tauchman et al. 2007), suggesting that JHBP upregulation in *SCD1* KD mosquitoes may be part of the signalling to suppress ovary development and PM synthesis.

Down-regulation of metabolism, replication and reproduction genes

Several genes encoding proteins that are functionally related to carbohydrate, lipid, protein and oxidative metabolism, reproduction and replication were significantly downregulated in *SCD1* KD mosquitoes (Fig. 4.1).

Carbohydrate and lipid metabolism:

A total of 116 genes in this category were significantly upregulated across time points; of those, 70.7% were downregulated. Amongst them were genes encoding *Vitellogenin* (*Vg*) and the major component of *Lipophorin* (*Lp*), *Apolipophorin* (*Apoll/I*), which showed >4-fold downregulation at 18 and 24 h PBM. *Vg* and *Lp* make up the majority of egg yolk proteins and are expressed at very high levels at 24 h PBM in response to hormones JH and 20-hydroxyecdysone (20E). Silencing of *A. gambiae* *apoll/I* has been previously shown to fully inhibit egg development (Vlachou, 2005; Mendes, 2008), in consistence with the *SCD1* KD phenotype reported in Chapter 3. The downregulation of *Lp* and *apoll/I* may be directly linked to inhibition of JH signalling as well as reduced levels of 20E due to presumably low levels of cholesterol (a 20E precursor) that is synthesized *de novo*; *SCD1* is important for triglycerol (TAG)cholesterol biosynthesis (Ntambi & Miyazaki 2004). Moreover, it may represent a feedback regulatory mechanism, whereby depletion of lipid droplets due to blockade of SFA to MUFA conversion signals for suppression of *Vg* and *Lp*, the major lipid carriers of the insect hemolymph. Importantly, silencing *Vg* and *Lp* potentiates *A. gambiae* immune response to rodent and human malaria parasites substantially limiting their infection

loads (Vlachou, 2005; Mendes, 2008; Rono, 2010). Taken together, these data suggest that *SCD1* KD differentially affects egg development in *A. coluzzii*.

Protein degradation/proteasome:

In this functional group, 46 genes were differentially regulated between *SCD1* KD and control mosquitoes, 67% of which were downregulated (Fig.4. 1). 41% of all downregulated genes were detected at 24 h PBM. Mosquitoes require a variety of proteolytic enzymes such as trypsins, serine proteases, carboxypeptidases and chymotrypsins to digest a recently acquired meal. Among downregulated genes were several exopeptidases (peptidase that release amino acid or dipeptide from peptide chain by catalysing the cleavage of the alpha peptide bond), including alanine aminopeptidase, serine protease S28, astacin, carboxypeptidase A1, and various trypsins, such as trypsin 7. Downregulation of alanine aminopeptidase was consistent among most of the time points: >3 folds at 0 h, >2 folds at 6 h PBM and >9 folds at 24 h PBM, respectively. Trypsin 7, serine protease S28, and carboxypeptidase A1 were significantly downregulated at 24 h PBM by >3, >5 and >4 folds, respectively.

Nevertheless, several peptidases were found to be upregulated in *SCD1* KD mosquitoes including peptidase M19 (>3 folds at 6 h PBM), peptidase S33 (>2 folds at 12 h PBM), trypsin 4 (>3 folds at 18 h PBM) and peptidase S60 (> 4 folds at 24 h PBM). It has been reported that enzymes like Trypsin 4, which are expressed early after a blood meal, may indirectly activate the transcription of the major endoproteolytic enzyme Trypsin 1 (Müller et al. 1995). Taken together, these data suggest that a complex network of proteolytic processes are involved in blood meal metabolism, which are differentially affected in *SCD1* KD mosquitoes contributing to the dramatic phenotypes reported in Chapter 3.

Oxidative metabolism:

A total of 35 genes belonging to the redox/apoptosis/detoxification category were differentially regulated in the *SCD1* KD mosquitoes (Fig.4. 1). The vast majority of these genes (83%) were downregulated, mostly at the 24 h PBM time point. Genes encoding cytochrome P450 proteins were significantly downregulated at 0 (CY314A1), 18 (CY302A1) and 24 h PBM (CYP12F1, CYP6P1, CYP9K1). Previous studies have shown that cytochromes P450 enzymes are key in all quantitatively significant pathways of cholesterol degradation, which maintain lipid homeostasis (Pikuleva 2006). Additionally, it is known that TAG cholesterol esters are substrates of carboxylesterase enzyme (Ross et al. 2010) and that *SCD1* KD blocks the downstream pathways for cholesterol TAG synthesis (Ntambi & Miyazaki 2004). Importantly, genes encoding carboxylesterases were also downregulated at

early time points: 0, 6 and 12 h PBM. Hence, it is possible that the scarcity of TAG cholesterol due to *SCD1* inhibition can lead to the downregulation of cytochrome P450 genes in a feedback manner.

Glutathione-S-transferase D7 (*GSTD7*) encoding-gene was over expressed at 18 and 24 h PBM. *GSTD7* has glutathione peroxidase activity (catalyses the reaction: 2 glutathione + hydrogen peroxide = oxidized glutathione + 2 H₂O.) and glutathione transferase activity (catalyses the reaction where glutathione conjugates with aliphatic, aromatic or heterocyclic group and a sulfate, nitrile or halide group) in *A. gambiae* (Ranson et al. 1998). GSTs together with P450s are major components of the detoxifying pathways of mosquitoes and an overexpression of isoenzymes, such as GSTA1 and GSTA2, attenuates lipid peroxidation in biological membranes (Yang 2001). In addition, lipid peroxidation affects mainly the polyunsaturated fatty acids (PUFA) of the cell membrane by removing electrons from them (Ostrea et al. 1985). Since *SCD1* KD distorts the SFA: MUFA ratio, it is possible, that *GSTD7* was upregulated in order to hinder lipid peroxidation and to maintain the homeostasis in *SCD1* KD mosquitoes.

Replication/transcription/translation/cell cycle:

Significant differential regulation was observed at 24 h PBM for 29 of 48 genes belonging to this category (Fig. 4.1). *Cyclin A*, an important regulator of cell cycle progression, was downregulated at 24 h PBM. The link between *SCD1* with cell cycle regulators is largely unknown. However, recent data indicates that *SCD1* blockage reduces AKT activity by dephosphorylation, with subsequent activation of glycogen synthase-kinase (GSK3- β), which in turn degrades cyclin D1 thereby inhibiting cell proliferation. AKT is a key component of the survival signalling pathway and GSK3 β is a downstream element of the AKT signalling pathway that is regulated by insulin (Doble & Woodgett 2003). It is suggestive that *SCD1* KD alters cell cycle progression in *A. coluzzii*.

Genes with altered expression across all time points

A total of 137 genes exhibited differential expression by at least 2-fold in *SCD1* KD versus control mosquitoes across all 5 time points, as illustrated in the heat map shown in Fig. 4. 2. Their putative function was analysed in order to better understand the biochemical and physiological effects of *SCD1* KD in the mosquito system independently of the blood meal. These genes are classified in functional classes and described below.

Lipid and carbohydrate metabolism:

The largest number of functionally annotated genes (19) belonged to the lipid and carbohydrate metabolism class (Fig. 4.2 and Table S1). Among downregulated genes 3-

hydroxy-3-methylglutaryl-CoA lyase (HMG-CoA lyase) was an essential enzyme releasing acetyl CoA by breaking down leucine and fats (Mitchell et al. 1998). It is hypothesized that HMG-CoA lyase downregulation is due to the increased flux of acetyl CoA, due to over-activation of β -oxidation. Another downregulated gene in this category was *UDP glucuronosyltransferase (UGT)*, which catalyzes the reaction in the glucuronidation process in insects to remove toxic compounds like fatty acid derivatives from body. In addition to the major role in inactivation and excretion of both endogenous and exogenous compounds, insect UGTs play important role in several processes, including cuticle formation, pigmentation and olfaction (Huang et al. 2008). It is suggestive that this reflects a feedback mechanism to prevent the removal of fatty acids.

Phospholipid scramblase (PLS1) is a plasma membrane-anchored enzyme responsible for the bi-directional translocation of phospholipids between the inner and outer leaflets to maintain cell membrane integrity. Disruption of this enzyme ultimately causes major defects in mitochondrial architecture, mass and transmembrane potential, and apoptotic responsiveness (Liu et al. 2003). *PLS1* was highly expressed in *SCD1* KD mosquitoes perhaps aiming to maintain the homeostasis of SFA/ MUFA ratios, which is important for membrane integrity. Overexpression of *PLS1* may additionally be related to induction of apoptosis in *SCD1* KD cells.

Replication/transcription/translation/cell cycle:

Downregulated genes in this class include an ATPase important in purine biosynthesis and the nuclear export factor RNA Binding Motif Protein 15 (RBM15) that is essential for efficient mRNA export from nucleus to cytoplasm (Zolotukhin et al. 2009). Together these data may indicate disruption of DNA and mRNA metabolism in *SCD1* KD mosquitoes (Fig. 4.2 and Table S1).

Signalling/ATPase/GTPase:

Insulin-like peptides (ILPs) have a variety of possible roles in mosquitoes including lipid and glycogen processing, immunity, reproduction and longevity (Wu & Brown 2006). In *Ae. aegypti*, ILPs family members including ILP3 stimulates blood metabolism and also ovaries to synthesize ecdysteroid hormone which induces the fat body to produce the yolk protein vitellogenin (Vogel et al. 2015). Importantly, *ILP2* is significantly downregulated across all 5 time points in *SCD1* KD mosquitoes (Fig. 4.2 and Table S1). These data may suggest the interruption of insulin mediated signalling in *SCD1* KD mosquitoes and perhaps defective ecdysteroid hormone synthesis. The phosphatidylinositol4-kinase type 2 (PI4K) synthesizes phosphatidylinositol4-phosphate (PI4P), an essential signalling and trafficking molecule.

PI4P is the main lipid determinant of Golgi and trans-Golgi network and helps to restore the acidic character of the plasma membrane and cell membrane fluidity that regulates PI4K activity (Boura & Nencka 2015). *PI4K* is significantly upregulated in *SCD1* KD mosquitoes. TOR is a member of the phosphatidylinositol4-kinase protein family which is closely related to PI4K protein. Therefore, it is suggestive that *SCD1* KD may alter signalling pathways such as the insulin and mTOR pathways.

Genes encoding odorant binding protein OBP13 and OBP26 are markedly upregulated across all 5 time points in *SCD1* KD mosquitoes. OBPs bind different odorant molecules and transport them to their respective olfactory receptors (Manoharan et al. 2013). OBPs of *Apis mellifera* and *Drosophila sechellia* have been shown to have strong affinity for saturated fatty acids like palmitate, stearate, octanoic acid and hexanoic acid (Xu et al. 2010; Briand et al. 2001). Therefore, the accumulation of saturated fatty acids due to the *SCD1* KD may trigger overexpression of *OBP13* and *OBP26* in mosquitoes.

Immunity/putative immunity/phagocytosis:

Haemocyte-enriched transcripts of the immune-related clip-domain serine proteases *CLIPD1* (Choi et al. 2012) and *CLIPB18* are upregulated in *SCD1* KD mosquitoes. The latter gene is shown to be involved in the mosquito melanisation cascade (Yassine & Osta 2010). A gene encoding a member of the lysosomal membrane proteins (LAMP) that are known to be involved in phago-lysosome formation as well as two CD80-domain encoding genes were also found to be upregulated across all time points. These responses suggest an inflammatory reaction in the absence of infection, perhaps due to increased concentrations of SFAs, or infection of the mosquito hemolymph. Upregulated genes include the *Tripartite Motif Containing 37 (TRIM37)*, which encodes a member of the tripartite motif-containing protein family. In addition to its diverse functions in development, TRIMs play crucial roles in innate immunity by inducing interferon expression (Rajsbaum et al. 2008). However, the specific function of TRIMs in insect system is yet to be discovered. Nevertheless, a member of the thioester-containing proteins, TEP12, shown to be induced as a response to wounding (Nsango et al. 2013), was downregulated highlighting the complexity of the immune response in *A. coluzzii* mosquitoes (Fig. 4.2 and Table S1). Together these data may indicate modulation in immune gene expressions regulation upon recognition of pathogen associated molecular patterns (PAMPs) and/or damage/danger-associated molecular patterns (DAMPs) in *SCD1* KD mosquitoes.

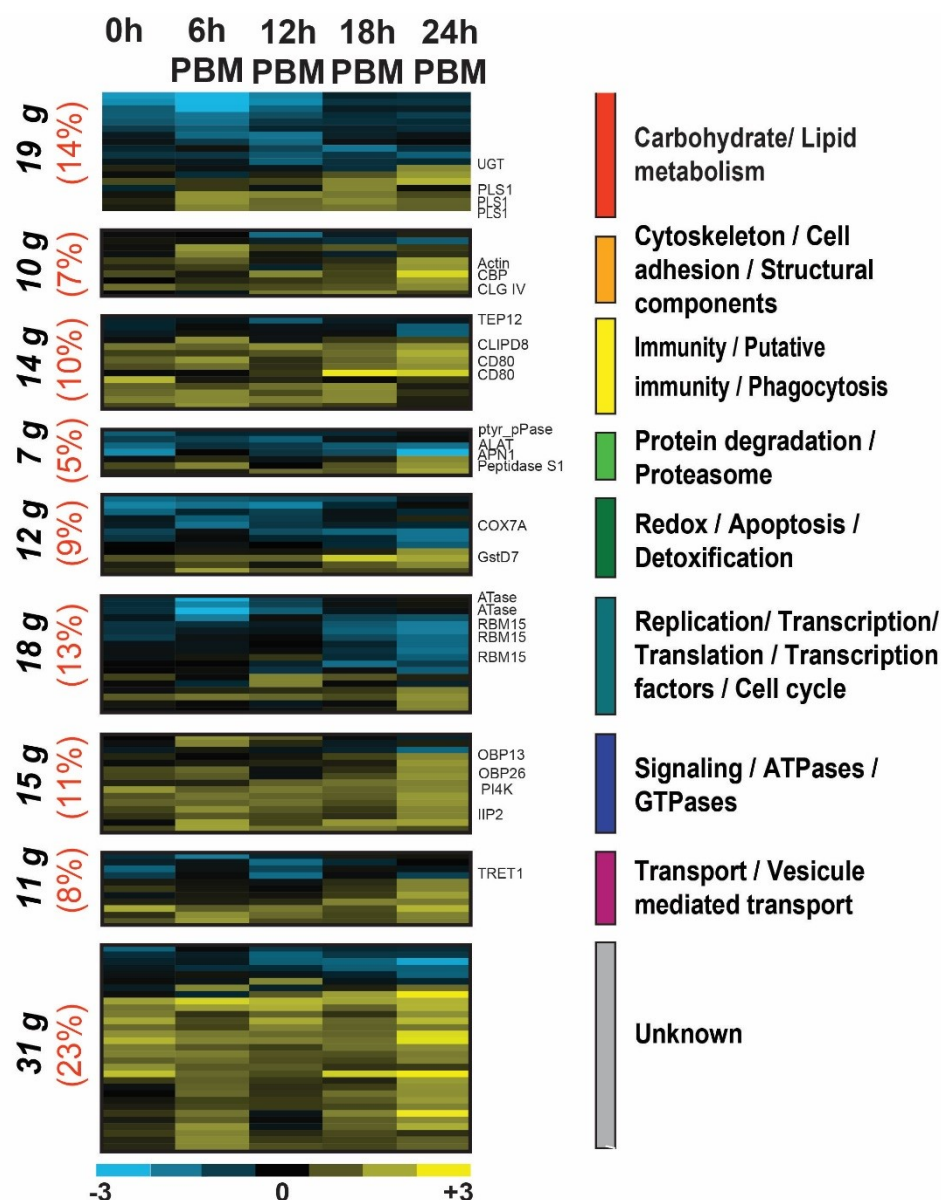


Fig. 4.2 Expression profiles of genes with altered expression across all time points in *SCD1* KD mosquitoes

A total of 137 genes showed differential expression across all 5 time points, by at least 2-fold, are shown in a heat map. Blue colour indicates downregulation and yellow colour indicates upregulation, as shown in the colour bar below the heat map. The data shown is the mean from three independent experiments. The number of genes classified in 8 functional categories or are of unknown function is shown together with the percentage of genes in each category. Here, g stands for gene.

Redox/ apoptosis/detoxification:

The detoxification gene *GstD7*, which plays an important role in protecting against cellular damage by controlling oxidative stress (Rngriti, 2005), was consistently upregulated in *SCD1* KD mosquitoes across all time points (Fig. 2 and Table S1). It has been demonstrated that *GstD7* is highly expressed in *Drosophila suzukii* as a response to exposure to the insecticide

malathion (Hamby et al. 2013). Cytochrome c oxidase polypeptide 7A (COX7A), the terminal component of the mitochondrial respiratory chain that catalyses the reduction of oxygen to water and can be allosterically inhibited by high ATP levels (Hüttemann et al. 2012), was downregulated across the five time points. Suppressed COX7A expression has been also revealed during acute inflammation (Hüttemann et al. 2012), supporting the hypothesis of a potent inflammatory response in *SCD1* KD mosquitoes.

Transport/vesicle mediated transport:

Trehalose transporter 1 (TRET1), which transports trehalose disaccharide from the fat body to hemolymph (Kikawada et al. 2007), was downregulated across the time course experiment (Fig.2 and Table S1). Silencing of Trehalose transporter gene reduces trehalose content in hemolymph and also shorten life span of *A. gambiae* under stresses like dessication or high temperature(Liu et al. 2013). Hence, it is likely that the protection against the stresses generated by *SCD1* KD in mosquito system, may be compromised in the *A. coluzzii*.

Cytoskeleton/cell adhesion/structural components:

This cluster of genes includes a downregulated *chitin-binding protein* (CBP), and upregulated *collagen IV* (CLG IV), a component of the basal lamina (Fig.2 and Table S1). Collagen alpha1(IV) is a probable contributor to a larger wound healing response in the *A. gambiae* midgut when it is infected with *Plasmodium yoelii* (Gare et al. 2003). Mosquito upregulates it's expression during the development of early oocyst of parasite. It is suggestive that peritrophic matrix (PM) synthesis was altered due to CBP downregulation and overexpression of CLG IV perhaps was induced as a response to wounding or compromised structure of PM and midgut epithelia in *SCD1* KD mosquitoes.

Protein degradation/proteasome:

This group of genes (Fig.4.2 and Table S1) includes *aminopeptidase N1* (APN1), a membrane associated protease, which was downregulated across 5 time points in *SCD1* KD mosquitoes. *Alanine aminotransferase* (ALAT) plays important role in effective disposal of excess nitrogen waste in blood fed female *Ae. Aegypti* (Mazzalupo et al. 2015). Accumulation of massive amount of uric acid in the midgut posterior region and interrupted blood metabolism were noticed in ALAT KD *Ae. aegypti*(Mazzalupo et al. 2015). In *SCD1* KD mosquitoes ALAT significantly downregulated across 5 time points. Cystatin like protein, traditional mediators of terminal bulk proteolysis in lysosome, encoding gene was also upregulated. In the tick *Ixodes scapularis* cystatin-like molecules in saliva plays prominent role in proteolytic cascades associated with an anti-inflammatory function(Kotsyfakis et al.

2006). Together these data may indicate disruption in protein metabolism in *SCD1* KD *A. coluzzii* mosquitoes.

Metabolomic profile of *SCD1* KD *A. coluzzii* mosquitoes

To examine the *SCD1* KD-induced biochemical changes, a GC-MS-based metabolomic analysis of deproteinized extracts of control *dsLacZ* injected and *SCD1* KD female mosquitoes at 0 (just prior to blood feeding), 18 and 36 h PBM was performed.

***SCD1* KD reduces total desaturase indices:**

Desaturase indices, the ratios of product to substrate of free fatty acids (saturated and unsaturated) metabolized by the *SCD1*, serve as a biomarker for the activity of the *SCD1* (Chajès et al. 2011; Ortinau et al. 2012). *SCD1* KD in female *A. coluzzii* mosquitoes caused a significant reduction in desaturase indices 16:1/16:0 (palmitolate to palmitate) at 18 and 36 h PBM and 18:1/18:0 (oleate to stearate) at all-time points (Fig. 3). These data suggest an overall increase of SFA content in the female mosquito body, and are consistent with those from studies in *SCD1* knock out in mice showing increased SFA content (Ntambi 1999).

***SCD1* KD alters aqueous metabolites:**

Significant differences in metabolite signals between experiment and control groups were evaluated by combining metabolite ratio cut-off 0.1 on a log2 scale. In total, the metabolomic analysis of aqueous metabolites revealed that 43 metabolites were differentially represented in *SCD1* KD compared to control mosquitoes (Fig. 4). In response to blood feeding, the levels of several extracted amino acids and many substrates of the TCA cycle (citrate, 0.6 fold at 36 h PBM; malate, 0.6 fold at 18 h and 1 fold at 36 h PBM; fumarate, 1.3 fold at 36 h PBM) increased while intermediates of glycolysis (D-glycerol-1P, 1.3 fold at 36 h PBM) and the pentose phosphate pathway (ribose 5-P, 1.4 fold at 36 h PBM; ribulose 5-P, 1.4 fold at 36 h PBM) decreased. The levels of D-glucose and D-glucose-6P remained constant.

Female mosquitoes largely convert protein-rich liquid meals into amino acids after blood ingestion. Consistent with these previous observations, small traces of amino acids were observed at 0 h PBM which significantly increased in *SCD1* KD mosquitoes at 36 h PBM compared to that of the control.

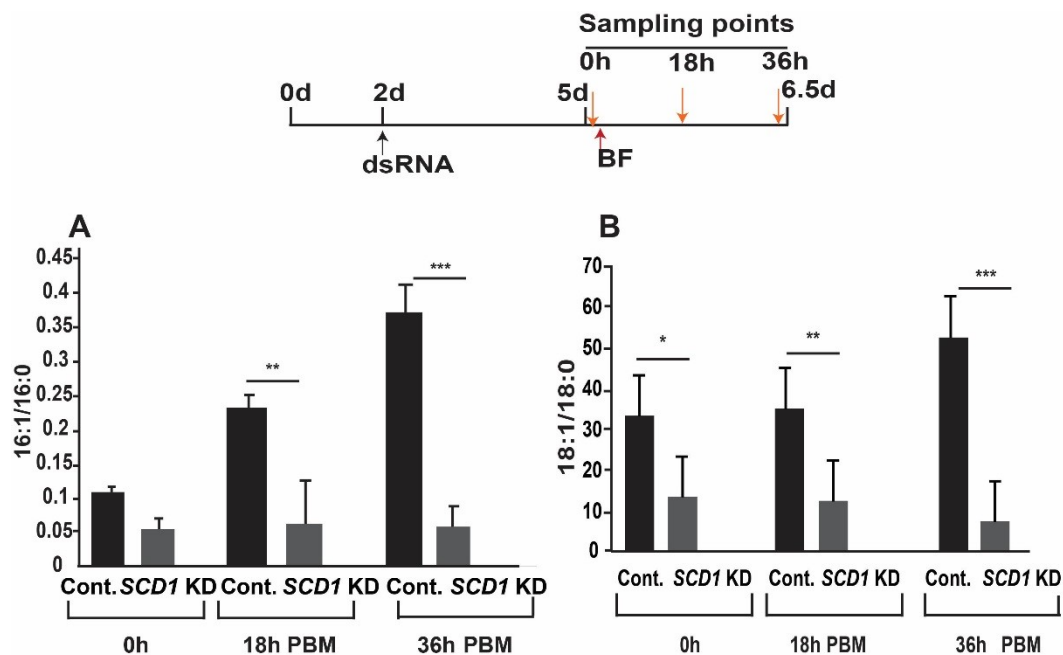


Fig. 4.3 SCD1 KD by dsSCD1 reduces total desaturase indices in female *A. coluzzii*

The graphs show desaturase indices that are the ratios of product to substrate of the fatty acids catalysed by SCD1 in control *dsLacZ* injected and SCD1 KD female mosquitoes. Indices at 3 time points were recorded: 0, 18 and 26 h PBM. The schematic at the top of the figure indicates the experimental design. Reported values are mean± SE of 5 independent biological replicates, where n=3 mosquitoes per group per replicate. *p<0.05, **p<0.001, ***p< 0.0001. Arbitrary units are presented on Y axis. (A) 16:1/16:0 desaturase indices (palmitolate to palmitate). (B) 18:1/18:0 desaturase indices (oleate to stearate).

During oogenesis, phenylalanine is preferentially channelled towards late protein synthesis in order to meet the need for egg cuticle hardening in *A. gambiae* (Fuchs et al. 2014). Interestingly, decreased levels of phenylalanine (1-2 folds) and its derivative tyrosine (1.5-2 fold) were observed at 18 and 36 h PBM. It can be therefore inferred that SCD1 KD leads to a perturbed conversion of phenylalanine to tyrosine and subsequent suppression of egg development. Increase in essential amino acids, such as isoleucine (0.7 fold at 36 h PBM) and valine (1.3 and 2 folds at 18 and 36 h PBM, respectively) may increase as a result of a feedback mechanism. Decreased levels (1.5-2 fold) of histidine, a major intracellular buffering agent in animal cells (Abe et al. 1985), was detected at 18 and 36 h PBM. Buffering agents maintain cellular pH and little changes in pH can cause either acidosis or alkalosis in the body with deleterious effects on metabolism. Hence, decreased levels of histidine may indicate imbalance in the pH system of SCD1 KD mosquitoes.

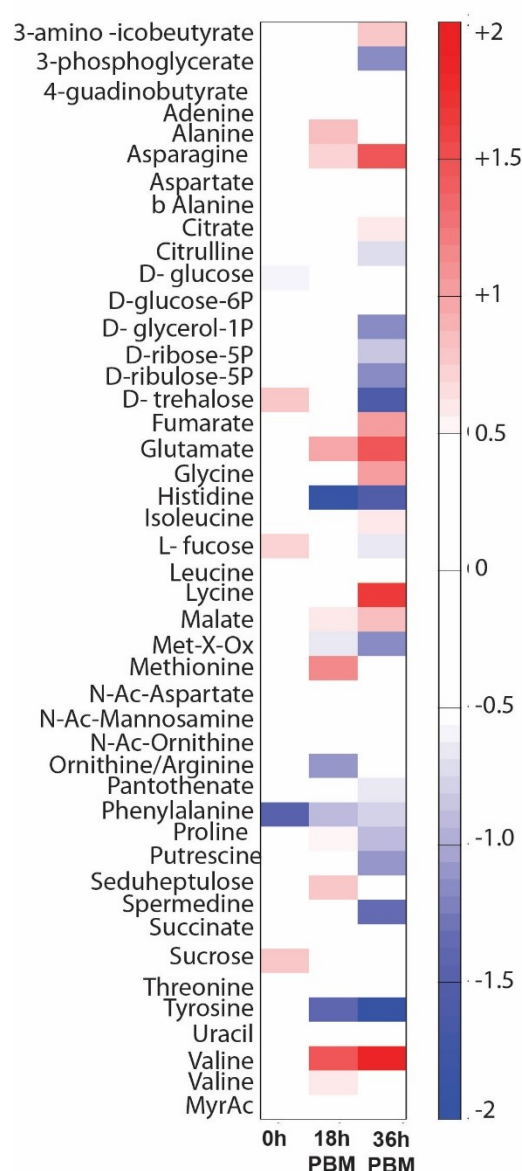


Fig. 4.4 Analysis of GC-MS mosquito metabolomes in response to *SCD1* KD at different time points

Metabolites which showed statistically significant ($p < 0.01$) changes in the experimental group compared to the control group are represented in this heat map. Reported values are the log₂ - fold changes in metabolite signal in *SCD1* KD mosquitoes. Five independent biological experiments were performed and $n = 3$ per group per experiment. 43 significantly regulated metabolites, compared to controls were identified in *SCD1* KD mosquitoes and most of them were observed at 36h PBM.

Impact of *SCD1* inhibition on mosquito metabolic pathways

In order to reconstruct the biochemical framework of the effects of *SCD1* KD on mosquito metabolic pathways following blood feeding, 16 genes identified by the transcriptomic analysis and 27 metabolites identified by the metabolomic analysis were mapped on

biochemical pathways using a KEGG pathway analysis (Fig. 5). Using vectorbase information genes those which are directly involved in different pathways were picked up (after functional analysis of microarray data set) and mapped on biochemical pathways. Of the 16 genes, 4 exhibited significant up or downregulation across all 5 time points (Fig. 2 and Table S1), while 12 showed significant up or downregulation at one or more time points.

The analysis revealed that silencing *SCD1* in female mosquitoes caused a coordinated suppression of the TCA cycle, glucose metabolism and fatty acid biosynthesis. Induction of genes involved in polyamine synthesis, the Leloir pathway and the lactate dehydrogenase (LDH) pathway were also observed. Among differentially regulated genes, acetyl CoA carboxylase (*ACC*), phosphoenolpyruvate carboxykinase (*PEPCK*), arginosuccinate synthase (*ASS*) and ornithine decarboxylase (*ODC*), produce proteins that occupy typical rate-limiting steps in the fatty acid biosynthesis, glyceroneogenesis, the urea cycle and polyamine synthesis pathway, respectively.

Fatty acid biosynthesis and the TCA cycle:

Silencing *SCD1* inhibits the conversion of SFA to MUFA, thereby generating lower palmitolate-to-pamitate and oleate-to-stearate ratios at 18 and 36 h PBM. This suggests an increased accumulation of SFA over time that hinders *ACC* activity though a well-known feedback mechanism resulting in a drop of malanoyl CoA that derepresses carnitine-palmitoyl-transferase (*CPT*) and in turn increases transport of acetyl CoA into mitochondria for β -oxidation (Kerner & Hoppel 2000). This upregulated β -oxidation in turn increases the reaction rate of many of the steps in the TCA cycle, thereby increasing flux throughout the pathway and resulting in higher accretion of TCA cycle intermediates over time (i.e. citrate, succinate, fumarate, malate), especially at 36 h PBM.

The significant increase in glutamate levels over time in *SCD1* KD mosquitoes, may suggest a compromised anaplerotic reaction, where glutamate converts to α -ketoglutarate. Succinate CoA ligase (*SUCL*-GDP forming) and Succinate CoA synthetase (*SUCS*-ADP forming) enzymes catalyse the only reversible reaction of the TCA cycle, where succinate and GTP or ATP are converted to succinyl-CoA and ADP or GDP, respectively. These enzymes are downregulated in *SCD1* KD mosquitoes suggesting higher levels of succinyl-CoA involvement in the TCA cycle (Ding et al. 2015). The elevated levels of isoleucine and methionine in *SCD1* KD mosquitoes compared to controls also suggest build-up of succinyl-CoA. Downregulation of *adenylosuccinate lyase* (*ADL*), which regulates cellular metabolism by controlling the amounts of fumarate, suggests feedback inhibition of increased accumulation of fumarate.

Urea cycle and polyamine synthesis pathway:

The genes encoding the *argininosuccinate synthase* (ASS) and *argininosuccinate lyase* (ASL), two key enzymatic components of the arginine biosynthetic pathway, where citrulline converts to arginine releasing fumarate, were downregulated in *SCD1* KD mosquitoes. It is plausible that the increased accumulation of fumarate leads to suppressed ASS and ASL expression (Husson et al. 2003). In *SCD1* KD mosquitoes, the intracellular levels of the polyamine putrescine were low compared to control mosquitoes. The apparent upregulation of *ornithine decarboxylase* (ODC) in *SCD1* KD mosquitoes, which converts ornithine to putrescine, and the low levels of ornithine (at 18 h PBM) compare to control may suggest further metabolism of putrescine initially to spermidine, which is also low in *SCD1* KD mosquitoes, and then through the glutathione detoxification pathways. An alternative explanation is that ODC transcripts do not translate to protein in *SCD1* KD mosquitoes. It has been shown that eIF-4E a regulator of ODC translation (Shantz 2004) is downregulated in *SCD1*^{-/-} mice through the mTOR pathway (D. Luyimbazi et al. 2010).

Glycolysis, gluconeogenesis and Leloir pathway:

Lower levels of sugar and downstream substrates of the glucose metabolism, such as glucose-6P, ribose-5P (an important substrate that controls the rate-limiting purine synthesis), trehalose and 3-phosphoglycerate, were observed in *SCD1* KD mosquitoes compared to controls across all 3 time points. Consistently, downregulation of the transcription level of two main enzymes of gluconeogenesis - Pyruvate carboxylase (PC) and Phosphoenolpyruvate carboxykinase (PEPCK) was also observed.

Upregulation of *lactate dehydrogenase gene* (LDH), which catalyses lactate synthesis, was also observed. Since lactate converts to glucose in order to maintain homeostasis of the cell during starvation, it is possible that *SCD1* KD mosquitoes activated this alternative pathway to compensate for glucose scarcity. However, the low levels of glucose and its downstream substrates in *SCD1* KD mosquitoes might activate the compensatory mechanism for glucose, by upregulating *galactokinase* (GALK), the first enzyme of the Leloir pathway, which converts galactose to glu-6P through several steps (Timson 2007).

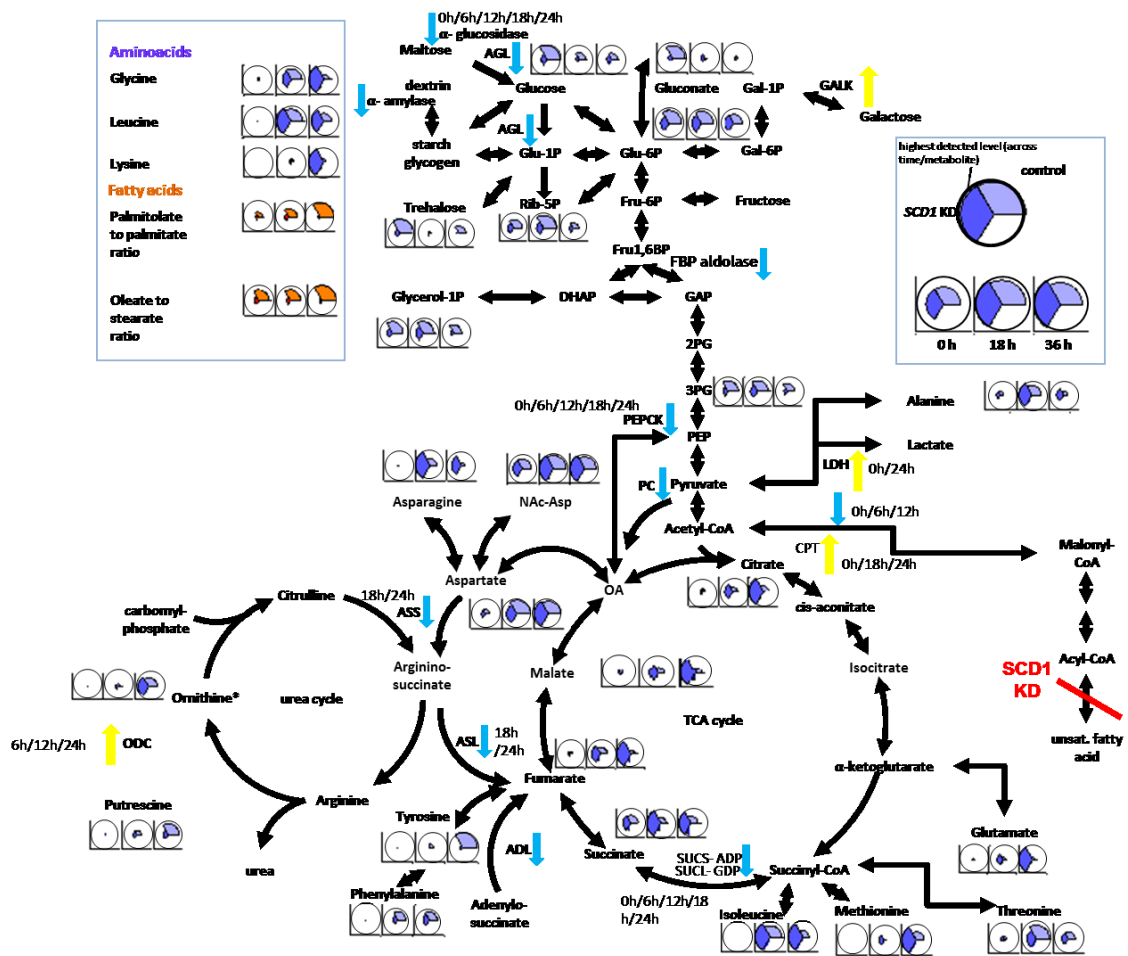


Fig.4. 5 Temporal impacts of *SCD1* KD on *A. coluzzii* metabolic pathways

The metabolic network that was reconstructed from the KEGG pathway included glycolysis (brown), fatty acid biosynthesis (orange), the TCA cycle and the urea cycle. Up and down regulation of genes in response to *SCD1* KD are shown by yellow and blue arrows. The extracted metabolite data was subjected to maximum values normalisation on a per metabolite basis and plotted as circle fragments. The radius of the circle is directly proportional to the fraction of the highest for the metabolite i.e. the black ring is equivalent to the highest level found for the compound across the three time points and sample types. Here, this representation of metabolite data allows observing changes between experiment and control mosquitoes over time in one graph.

Downregulation of fructose-bisphosphate aldolase enzyme, which converts fructose 1, 6-BP to glyceraldehyde-3-phosphate (GAP), a precursor of dihydroxyacetone phosphate (DHAP), might cause the lower level of glycerol 1-phosphate. It is plausible that 4- α glucanotransferase, amylo- α -1,6-glucosidase, α amylase, and α glucosidase,

encoding genes related to glycogen synthesis, were downregulated due to the depleted level of their precursor, the UDP glucose (Estela L Arrese & Soulages 2010).

Amino acid metabolism:

Higher levels of asparagine at 18h and 36h PBM and insignificant changes in the level of aspartate in *SCD1* KD mosquitoes, compare to controls, may suggest the compromised conversion of asparagine to aspartate. It is also possible, that the compensatory pathway, where Nac-Asp converts to aspartate and vice versa, was not affected in *SCD1* KD mosquitoes. Increased glutamate that is converted to α -ketoglutarate, a substrate of the TCA cycle, was observed in *SCD1* KD mosquitoes compare to control at 36 h PBM. In addition to glutamate, higher levels of methionine and isoleucine were detected at 36 h PBM. Both methionine and isoleucine convert to Succinyl-CoA, another substrate of the TCA cycle. Increase in essential amino acids such as methionine and isoleucine may be the result of a feedback mechanism of over activation of the TCA cycle. However, levels of phenylalanine and tyrosine were lower in *SCD1* KD compared to control mosquitoes.

SCD1 blockade modulates immune gene expression

As described above, *SCD1* KD leads to significant induction of immune responses suggesting either a perturbation of the mosquito immune homeostasis or infection as a result of compromised gut epithelial integrity. Inhibition of *SCD1* has been shown to alter cellular functions by regulating inflammation and stress in several kinds of cells and tissues, such as adipocytes, liver, macrophages, aorta, skin, myocytes, B cells, and endothelial cells in both human and mouse models. Therefore, it was of interest to investigate the induction of immune response in order to get further insight into *SCD1* function in mosquitoes. To achieve this goal, immune genes that were significantly upregulated in at least one time point were analysed.

The vast majority of immune genes upregulated in *SCD1* KD compared to control mosquitoes, in particular after a blood meal, could be classified as part of a systemic immune response (Fig. 6A). The gene encoding TEP3, a member of the thioester-containing protein family, was amongst upregulated genes. TEP3 is paralogous to the main effector of the complement pathway, TEP1, but is thought to not carry a thioester motif and therefore have a regulatory rather than effector function. Like TEP1, TEP3 binds to the putative receptor/adaptor complex LRIM1/APL1C and is involved in responses against bacteria and malaria parasites (Schnitger et al. 2007; Dong et al. 2006b; Povelones et al. 2011). Another component of the complement pathway upregulated in *SCD1* KD mosquitoes is CTLMA2, a member of the C-type lectin protein family. CTLMA2 is transcriptionally induced in response

to infection an is thought to promote pathogen lysis and/or phagocytosis and negatively regulate the melanisation reaction (Schnitger et al. 2009).

Many of the regulators of the mosquito systemic response including complement and melanisation (which itself is thought to be a function of complement) belong to the family of CLIPs. Indeed, 25% of the differentially regulate immune genes belonged to the CLIP family, including *CLIPA5*, *CLIPA8*, *CLIPB11*, *CLIPB12*, *CLIPB14-20* and *CLIPD1*; all but *CLIPB20* were upregulated (Figure 4.6A). Melanisation is an instant immune response of arthropods where organisms form dense melanin coating around pathogens or on the wound area (Yassine et al. 2012). Several of the CLIPs bear serine protease catalytic activities and are thought to directly promote amplification of the melanisation cascade, while others are predicted to not have an enzymatic activity and function as regulators. *CLIPA8* is thought to be a core regulator of melanisation, promoting but not directly catalysing activation of the melanisation effector enzymes pro-phenoloxidasases (PPOs) against bacteria, malarial parasites and fungi (A. K. D. Schnitger et al. 2007; Yassine et al. 2012). Importantly, PPO3 was also upregulated at 24h PBM in *SCD1* KD mosquitoes. Two other important and enzymatically active CLIPs, *CLIPB14* and *CLIPB15*, are also upregulated in response to bacterial challenges and are involved in antibacterial defence although their function is yet poorly understood (Volz 2005). The *Ae. aegypti* ortholog of *CLIPD1* is shown to promote hemocyte mediated immunity in response to infections by the filarial nematode *Brugia malayi* (Juneja et al. 2015).

The scavenger receptor *SCRQB3* is also thought to be involved in melanisation. It is induced in response to injury and the associated opportunistic infections as well as upon injection of Sephadex beads, which are subsequently melanised (Warr et al. 2006).

The fibrinogen related proteins, FREPs, play key role in mosquito humoral and systemic responses and are thought to have complementary and synergistic functions (Dong & Dimopoulos 2009). Strong upregulation in *SCD1* KD mosquitoes was observed for *FREP19*, *FREP21*, *FREP24*, *FREP32*, *FREP59* and *ficolin A*.

MD-2-like genes encode proteins that recognise lipopolysaccharide (LP) molecules (Bryant et al. 2010; Visintin et al. 2006). The founding member of this family acts synergistically with TLR4 to trigger the expression of inflammatory cytokines in response to bacterial LPS. However, there is no evidence has been found in insects that Lipid- binding MD2-like proteins (MLs) are involved in inflammatory responses. MLs in *A. gambiae* are found to confer resistance to both rodent and human malaria parasite species, especially *ML1* that regulates resistance to *P. falciparum* (Dong et al. 2006a). *A. gambiae* also upregulates MD2-like genes (*ML1/9*) in response to O'nyong-nyong virus infection (Waldock et al. 2012). Two

members of this family (*ML4* and *ML7*) were strongly upregulated in *SCD1* KD *A. coluzzii*. Therefore, It might be possible that mosquitoes upregulate *ML4* and *ML7* to recognize abnormal and excessive saturated fatty acids and to active inflammatory reactions. Though, it needs thorough investigations to establish the relationship between the activity of *MLs* and inflammatory responses in mosquitoes.

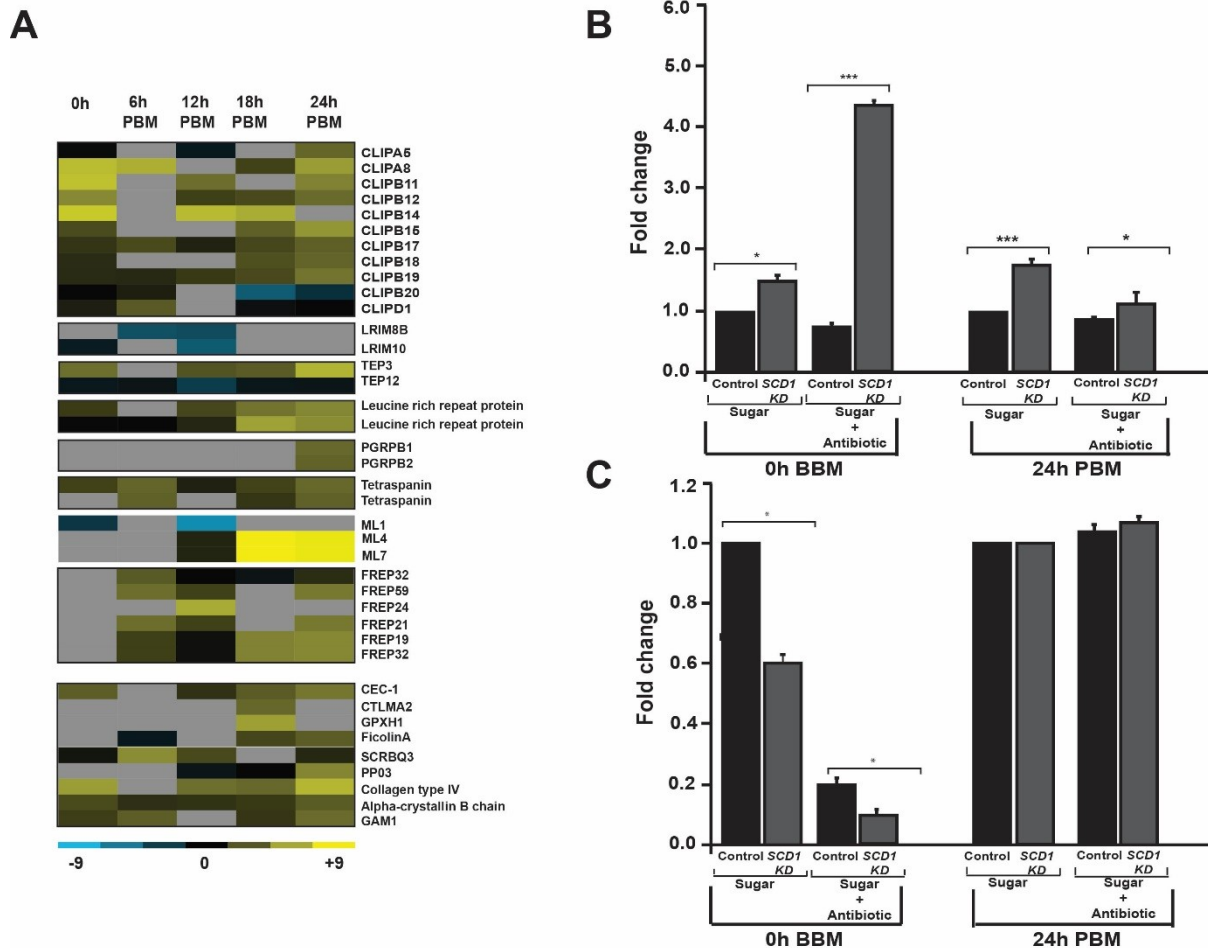


Fig. 4.6 Expression of immune genes in *SCD1* KD mosquitoes

(A) Heat map representation of microarray data about immune gene expression in *SCD1* KD vs. control mosquitoes. Immune genes are from the Immune DB database and manual curation. Significant expression differences across 5 time points are only presented on a spectrum ranging from downregulation (blue) to upregulation (yellow). Expression is $\log_2$ - fold changes in response to *SCD1* KD. Black and grey colours indicate insignificant/low variability and no data available, respectively. (B) *Cec-1* expression in antibiotic-treated *SCD1* KD mosquitoes at different time points. (C) The bacterial load in the midguts of sterilized antibiotic treated *SCD1* KD mosquitoes at 0 and 24h PBM. Bacterial load was estimated by using broad range 16s bacterial primers using qRT-PCR. Reported values are the mean $\pm$ SE of three independent biological experiments and $n = 10$ per group per experiment.

The antimicrobial peptides cecropin 1 (*CEC1*) and gambicin1 (*GAM1*) were also upregulated in *SCD1* KD mosquitoes. These peptides are shown to be effective against bacterial infections (Dong & Dimopoulos 2009). The hallmark of the humoral defence in *A. gambiae* is the systemic secretion of antimicrobial peptides (AMPs) such as CECs, four defensins, one *GAM1* and one attacin by fatbody (Christophides et al. 2004). Among all these AMPs, *CEC1* has a broad spectrum of antibacterial (both gram positive and gram negative), antiparasitic activity, as well as antiviral activity against CHIKV, DENV and HIV. Therefore, it is worth checking *CEC1* expression for responses against midgut bacteria to understand the immune response dynamics in *SCD1* KD mosquitoes.

***CEC1* induction in *SCD1* KD mosquitoes is independent of the midgut microbiota**

There are two possible explanations of the observed upregulation of immune-related genes in *SCD1* KD mosquitoes, most of which are involved in systemic immune reactions of the hemolymph. The first hypothesis is that over-activation of the immune system is caused by gut microbiota or their immune inducers gaining increased access to pattern recognition receptors on the midgut epithelium or the hemolymph due to the loss of the integrity of the peritrophic membrane and the gut epithelial barrier. The second hypothesis is that the increased concentrations of SFAs or purinergic molecules such as ATP, ADP trigger auto-inflammatory signalling. In invertebrates, humoral substances called (DAMPs released by damaged cells or tissues activate cellular receptors through signalling pathways leading to downstream inflammation. Parasite invaded compromised mosquito epithelial cells release intracellular DAMPs contents like intracellular nucleotides, reactive oxygen species, extracellular purinergic molecules, nucleic acids, and heat shock proteins, which promote immune responses. Effects of inflammation includes wound sealing, replacement of damage cells, and the phagocytosis of the damaged cells (Moreno- García et al. 2014).

To investigate the first hypothesis, control (*lacZ* injected) and *SCD1* KD mosquitoes were treated with antibiotics solution provided through the sugar meal from the time of emergence in order to clean the gut from its resident microbiota. After 5 days of antibiotic treatment, mosquitoes were fed on human blood through a membrane feeder. Mosquitoes from each group were sampled at 0 (just prior to blood feeding) and 24 h PBM and RNA was extracted. The expression of *CEC1*, which was found to be upregulated across time points in the microarray experiments, was examined by qPCR. The load of midgut bacteria was also quantified by qPCR of the 16S rRNA. Three independent biological replicates were performed.

In consistence with the microarrays observations, the expression of *CEC1* at 0 h PBM was significantly higher (1.5 fold, $p < 0.01$) in *SCD1* KD mosquitoes that received a sugar meal without antibiotic compared to controls. An even stronger induction (4.5 fold, $***p < 0.0001$) was observed in antibiotic-treated *SCD1* KD compared to control mosquitoes (Fig. 6B). Overexpression of *CEC1*, albeit less pronounced than at 0 h PBM, was also observed at 24 h PBM in both antibiotic untreated (1.9 fold, $***p < 0.0001$) and untreated (1.2 fold upregulation, $*p < 0.01$) mosquitoes. No difference in *CEC1* expression was observed between antibiotic treated and untreated control and *SCD1* KD mosquitoes at 24 h PBM. The upregulation of *CEC1* in both antibiotic treated and untreated control and *SCD1* KD mosquitoes suggests that the midgut microbiota play no important role in the increased immune response of *SCD1* KD mosquitoes. On the contrary, the substantially higher *CEC1* induction in antibiotic-treated compared to untreated *SCD1* KD mosquitoes suggests that the microbiota are playing an inhibitory role to immune signalling that is likely to be triggered by the distorted SFA:MUFA ratio, perhaps through tolerance pathways.

Microbiota density was also estimated in order to measure the efficiency of antibiotics in cleaning up the mosquito midgut. The microbiota load had increased at 24h PBM in both cases (only sugar fed and antibiotic treated mosquitoes) (Fig.6C).

Discussion

The investigation of the associations between gene expression, metabolic features and phenotypes in living organisms is a powerful approach in the drive for understanding the function of a particular gene. Having this in mind, a DNA microarray and metabolome analysis were performed in order to interpret the dramatic functional and phenotypic consequences, i.e. shorter life span, thorax full of blood, and undeveloped eggs, observed in *SCD1* KD mosquitoes. An additional goal was to get a global idea about the genotypic and biochemical changes occurring in *SCD1* KD in mosquitoes.

SCD1 is a major rate-limiting enzyme which converts SFAs (stearic and palmitic acid) to MUFAs (oleic and palmitoleic acid) and is involved in many biological processes, such as cell membrane fluidity preservation, signal transduction, replication, cell growth and the cell cycle (Ntambi 1999). A significant number of genes (601) were differentially expressed before and after blood meal ingestion by *SCD1* KD mosquitoes. Blood meal triggers a complex program of gene expression in a few hours and that includes the activation and/or upregulation of several genes required for the induction of physiological, morphological and hormonal changes in mosquitoes (Sodja et al, 2007). At 6h PBM, the number of regulated

genes (141 genes) almost doubled in comparison to those of 0h, which may suggest that the blood meal aggravated the consequences of *SCD1* KD in the mosquitoes' systems.

The induction of lipotoxicity via excess SFA in the *SCD1*-inhibited cancer cell model leads to cell dysfunction and mortality, though an increase in basal apoptosis (Scaglia et al., 2009). Previous studies have demonstrated that excessive accumulation of palmitic acid, the most common SFA in organisms, induces apoptotic cell death by causing ER stress (Gu et al., 2010; Park et al., 2014; Zhang et al., 2012). In this project, significant mortality was observed in *SCD1* KD mosquitoes between 24 and 48h PBM, and first death was recorded at 18h PBM (Chapter 3). This phenotype is consistent with the metabolite data taken from in *SCD1* KD mosquitoes; it showed a significant reduction of desaturase indices (16:1/16:0; 18:1/18:0) at 18 and 36h PBM. Since a reduction of desaturase levels suggests an eventual accumulation of excess SFA in cells (Matsui et al. 2012), it is likely that lipotoxicity, owing to excess SFA, is one of the major causes of mosquito death, both in sugar-fed and blood-fed mosquitoes. The above mentioned data, from this particular project, shows agreement with previously reported studies, where loss of *SCD1* activity lead to significant excess accumulation of SFAs and a reduced viability in different stages of *Plasmodium* parasites as well as 100% mortality in chick embryos (Gratraud et al., 2009; Austic, Hill, & Wilson, 1971).

Chitin is the most widespread amino polysaccharide, composed of N-acetylglucosamine residues, in nature. Chitin is the fundamental component of the PM, which serves as a permeability barrier between the food bolus and the midgut epithelium, and which expedites digestive processes and defends the brush border from mechanical disruption and attacks by toxins and pathogens (Tellam, 1996). In insects, the biosynthesis of chitin pathway starts with trehalose, the chief hemolymph sugar in most of the insects, and ends with N-acetylglucosamine residues (Cohen 2001). During periods of starvation, some insects completely cease peritrophic matrix synthesis. Moreover, a large number of studies demonstrated that *SCD1* inhibition improves insulin activity in the mouse liver, and is accompanied with a lower level of postprandial glucose and insulin (Flowers 2006). Significant downregulation of *CBD* encoding gene, at 0 (>2 folds), 12 (>2 folds), and 24 (>5 folds), and consistently lower levels of glucose, at 0, 18 and 24h PBM, were observed in *SCD1* KD mosquitoes. Additionally, steady downregulation of *IIP2* across the 5 time points was also noticed. Considering all the published data, it can be hypothesized that, during the post-injection recovery time (3 days), *dsSCD1* injected mosquitoes may activate the insulin pathway which is then followed by a depletion of glucose levels (no data available). Thus, lower levels of sugar may hinder chitin synthesis in a feedback manner in *SCD1* KD mosquitoes, which may lead to a delicate PM. Therefore, it can be inferred that, these

findings may be linked with the dramatic phenotype of blood meal-leakage into the mosquito body cavity, which is due to a fragile PM in *SCD1* KD mosquitoes at 24h PBM.

In *A. gambiae*, dramatic distension of the midgut by a remodelling of epithelial cells, after blood meal ingestion, is associated with the over-expression of the actin gene (Sodja et al. 2007). It has become evident that, *SCD1* inhibition results in a shift from unsaturation towards saturation in the fatty acyl chains in cell membranes and a concomitant increase in stiffness (X. Liu et al. 2011). Upregulation of actin gene expression in *SCD1* KD mosquitoes may be the compensatory response of the mosquito system aimed at overcoming the rise in mechanical pressure caused by a rigidity of the cell membrane and blood bolus.

In the egg development process, lipids and major egg yolk proteins are synthesized by the fat body and are transported to the ovaries. Vitellogenins - the precursors of lipoprotein and phosphoprotein - make up the major part of egg yolk proteins in mosquitoes (Chen et al. 2007). The downregulation of lipid transport protein encoding genes, such as vitellogenin and apolipoprotein, was observed in *SCD1* KD mosquitoes. In opposition to this data, it has been demonstrated that, vitellogenin is transcribed at a very high level in anautogenous mosquitoes at 24h PBM (Chen et al. 2007). In female mosquitoes, JH plays a pivotal role in the regulation of *YPP* genes. It has been demonstrated that, JH treatment revives the nutrient signalling pathway in the fat body, vitellogenesis and the effective development of eggs after blood meal in malnourished *Ae. aegypti* (Hansen et al. 2014). In *SCD1* KD mosquitoes, *JHBP* was overexpressed, suggesting a lower level of JH (Tauchman et al. 2007). It is possible that, the expression of yolk protein precursors may be affected by the overexpression of *JHBP* and lead to subsequently undeveloped ovaries in *SCD1* KD mosquitoes. Moreover, in mosquitoes, several insulin-like proteins, such as *IIP2*, are known to induce ovaries to synthesize ecdysteroid hormone, which in turn stimulates the fat body to synthesize yolk protein for egg development (Vogel et al., 2015). Downregulation of *IIP2* in *SCD1* KD mosquitoes may also be involved in the formation of undeveloped eggs by affecting the ecdysteroid synthesis process in *SCD1* KD mosquitoes. Moreover, *ALAT* gene was downregulated in *SCD1* KD mosquitoes. Deficiency of *ALAT* genes in female *Ae. aegypti* causes delay in blood digestion accompanied with delayed oviposition and reduced egg production and accumulation of toxic uric acid (Mazzalupo et al. 2015). Hence, it is likely that this gene also contribute in the formation of immature eggs. mTOR signalling pathway has crucial role in egg development since mosquito senses amino acids via mTOR and exerts signal for *YPP* expression. It is possible that mTOR signalling pathway is also affected by *SCD1* KD *A. coluzzii*.

In addition to differential gene expressions, lower levels of phenylalanine and tyrosine were observed at 18 and 36h PBM in *SCD1* KD mosquitoes. Phenylalanine hydroxylase (PAH), which converts phenylalanine to tyrosine, shows upregulation at the time of egg formation in mosquitoes, suggesting its potential role in yolk protein synthesis. Inhibition of PAH, leading to a low level of tyrosine, causes small sized ovaries in blood fed female *A. gambiae* (Fuchs et al. 2014). These altered gene expressions and levels of metabolites correspond to an undeveloped ovaries phenotype in *SCD1* KD mosquitoes at 24h PBM.

SCD1 is linked to diverse metabolic processes such as lipogenesis, fatty acid oxidation, insulin signalling, and carbohydrate metabolism in mice (Nguyen et al. 2014). It has been demonstrated, in different animal models, that *SCD1* inhibition leads to the accumulation of SAF, which in turn suppresses ACC through a well-known feedback mechanism, ultimately resulting in a drop of malonyl-CoA levels (Palton, 2007). Malonyl-CoA derepresses carnitine-Palmitoyl β -Transferase (CPT) and increases transport of acetyl CoA into the mitochondria for β -oxidation. In a manner consistent with these findings, the current study of *SCD1* KD also lead to downregulation of the *ACC* gene and upregulation of the *CPT* gene at 5 different time points in the *A. coluzzii* model. An accumulation of different intermediates of the TCA cycle, such as citrate, succinate, fumarate, and malate, at 36h PBM was noticed in *SCD1* KD *A. coluzzii*. Excess entry of acetyl coA molecule into the TCA cycle (or of any of the intermediates of the TCA cycle) increases all the other intermediates, since one is converted into the others (Stryer, 1995; Bu & Mashek, 2010). This upregulation of beta oxidation, accompanied with the activation of the TCA cycle in *SCD1* KD mosquitoes, shows agreement with a previous study, where hepatic *SCD1* KD (via RNA interference) in obese mice, demonstrated a reduction of the lipid content through over activation of beta oxidation (Xu et al. 2007). It has been demonstrated that, insulin-mediated glucose uptake is increased in *SCD1*^{-/-} mice (Rahman et al. 2003). Similarly to this data, lower levels of glucose were observed in *SCD1* KD mosquitoes at 0, 18 and 36h PBM. In addition to this, downregulated *IIP* gene expression was also observed at all 5 time points in *SCD1* KD mosquitoes. Therefore, it is likely that during the 3 days of post-injection recovery time in *SCD1* KD mosquitoes, insulin-mediated glucose uptake might have increased and resulted in the depletion of glucose levels, even at 0h, and that *ILP* transcription was downregulated through a feedback mechanism. It is hard to conclude that the downregulation of *IIP2* was a result of glucose depletion, since there is no data available for *IIP2* gene expression, or on the glucose levels before 0h. Therefore, there is another possibility - that of having an independent mechanism of insulin signalling for glucose uptake in mosquitoes. It has been shown that, malonyl CoA depletion encourages glucose uptake and use in mice, though a

mechanism which is independent of known insulin signalling pathway mechanisms (Muoio & Newgard 2008).

Glucose production (~75%), gluconeogenesis, and glycogenolysis all show a marked decrease, accompanied by downregulation of *PEPCK* gene expression, in antisense oligodeoxynucleotide-mediated *SCD1* KD mice (Roger, 2006). Similarly to this study, glucoregulatory enzymes, such as PEPCK and glycogenolysis enzymes, showed a decrease in transcriptional expression in *SCD1* KD mosquitoes.

Studies have shown that, the inhibition of β -oxidation inhibits AKH-induced release of trehalose in two cockroach species (Arrese & Soulages 2010). Similar results have been found in this study. The level of trehalose - the major carbohydrate storage molecule - was significantly lower at 36h PBM and *TRET1*, which transports trehalose and disaccharides from fat body to haemolymph (Kikawada et al. 2007), was downregulated (≥ 0.8 -2 folds) in *SCD1* KD mosquitoes.

The TCA cycle is the central metabolic pathway, and other metabolic pathways are closely linked to it. Over-activation of the TCA cycle causes the waste of large amounts of metabolic energy in the overproduction of reduced coenzymes, such as NADH and ATP. A reduced amount of ADP - the major substrate which gets converted to ATP - causes accumulation of precursor NADH, which in turn can inhibit a number of enzymes involved in the TCA cycle, including Succinate CoA ligase and Succinate CoA synthetase (Ivannikov & Macleod 2013). Therefore, it is likely that the genes for SUCL- GDP forming and SUCS- ADP forming enzymes and ADL, in *SCD1* KD mosquitoes, were downregulated in the manner of a feedback mechanism. It has been reported that, elevated levels of glutamine, alanine and a raised lactate/pyruvate ratio are associated with a deficiency of the SUCL-GDP forming gene in the mitochondrial hepatoencephalomyopathy of newborns (Van Hove et al. 2010). In agreement with this evidence, increased level of glutamate, alanine (at 36h PBM) and upregulation of the lactate dehydrogenase enzyme encoding gene, which converts pyruvate to lactate, were observed at 0 and 24h PBM in *SCD1* KD mosquitoes.

Downregulation of *ASS* and *ASL* genes - both key enzymes of the urea cycle, which catalyze the condensation of citrulline and aspartate to form argininosuccinate, and break argininosuccinate, to release fumarate and arginine respectively - were observed in *SCD1* KD mosquitoes. It has been shown that, both in vascular endothelial cell cultures and in whole animal studies, insulin upregulates *ASS* transcription to support nitric oxide production (Haines et al. 2012). It has also been shown that, insulin receptor signalling, and eventual insulin secretion, increased in *SCD1* knock-out mice (Rahman et al. 2003). Considering

these two pieces of evidence, it can be hypothesized that, downregulation of the *IIP 2* gene (mentioned above) may regulate the expression of ASS in *SCD1* KD mosquitoes.

Significant reduction of putrescine in *SCD1* KD mosquitoes suggests abnormalities in the cellular processes as it is essential for fundamental processes, such as stabilization of chromatin and the cytoskeletal structure, translation, transcription, semiconservative DNA replication, and the protection of cells from DNA damage (Mandal, 2012). However, the transcriptional level of *ODC* - a key enzyme of the polyamine biosynthetic pathway - was higher in *SCD1* KD mosquitoes than control mosquitoes. It has been shown that, transcripts of the *ODC* gene are regulated at the translational phase by Eukaryotic initiation factor 4E (eIF4E), a positive regulator of *ODC*. Moreover, inhibition of eIF4E decreases *SCD* expression in breast cancer (Luyimbazi et al. 2010; Shantz, 1999; Anthony, 2006; Luyimbazi, 2010). Hence, it is possible that, *ODC* transcripts were not translated, owing to the inactivation of eIF4E. Moreover, downregulation of *ATase* - a key enzyme of purine synthesis (Yamaoka et al. 2001) - and *RBM15* - essential for efficient mRNA export from nucleus to cytoplasm (Zolotukhin et al. 2009) - suggest *SCD1* KD-mediated disruption in DNA/RNA synthesis and the export system in mosquitoes.

Mosquitoes require a variety of proteolytic enzymes such as trypsins, serine protease, carboxypeptidase and chymotrypsin to digest a recently acquired meal. Proteolytic enzyme *APN1* shows peak activity at 24h PBM in *A. gambiae* (Dana et al. 2005). Conversely to this data, expression of *APN1* was significantly downregulated along with aminotransferase in *SCD1* KD mosquitoes. Trypsin genes, such as Trypsin 4 and Trypsin 7, which is expressed prior to blood feeding in female mosquitoes, are downregulated following a blood meal and not expressed again at levels detectable by RT-PCR, until 28 hours post-blood meal in *A. gambiae* (Müller et al. 1995). Similar to this study, downregulation was observed in *SCD1* KD mosquitoes compared to the controls. Trypsin 4 appears in the *A. gambiae* gut after 20h PBM, and reaches maximal expression by 48h PBM (Müller et al. 1995). Interestingly, Trypsin 4 was significantly upregulated at 18h PBM in *SCD1* KD mosquitoes. Therefore, it is likely that, *SCD1* KD mosquitoes upregulated their expression in order to synthesize more Trypsin 1 and to expedite blood digestion, as it has been reported that Trypsin 4 may indirectly activate the transcription of *Trypsin 1* (Müller et al 1995). These changes, in the expression of proteolytic genes in *SCD1* KD mosquitoes, may point to an incomplete protein digestion. The disruption in blood metabolism may correspond with the phenotype of a bigger and dark blood bolus, observed in *SCD1* KD mosquitoes at 24h PBM.

Interestingly, most of the genes in the “redox/apoptosis/detoxification” category were significantly downregulated among the 5 time points. Cytochrome P450 (P450s) enzymes

initiate all quantitatively significant pathways of cholesterol degradation used to maintain lipid homeostasis (Pikuleva 2006). Another study has shown that, triglycerols (TAG), cholesterol are substrates of the carboxyltransferase enzyme - a detoxifying enzyme which protects the system from esters and amide compounds (Ross et al. 2010). Genes, encoding for the cytochrome P450 protein (CYP314A1, CYP302A1, CYP12F1, CYP6P1, CYP9K1), were significantly downregulated at 0h, 18h and 24h PBM in *SCD1* KD mosquitoes. Carboxyltransferase-encoding gene (COE130) was also markedly downregulated at early time points such as 0, 6 and 12h PBM in *SCD1* KD mosquitoes. Since *SCD1* KD blocks the downstream pathways for cholesterol TAG synthesis (Ntambi & Miyazaki 1999), it is possible that the scarcity of TAG cholesterol, may be due to *SCD1* inhibition in mosquito, which lead to the downregulation of cytochrome P450 genes in a feedback manner.

It has been demonstrated that, overexpression of isoenzymes of GSTs (such as GSTA1 and GSTA2) attenuate lipid peroxidation in biological membranes (Yang 2001) and that lipid peroxidation affects mainly the poly-unsaturated fatty acids (PUFA) of the cell membrane, by stealing electrons from them (Ostrea et al. 1985). Similarly to these studies, *GSTD7* - a major component of the detoxifying pathways of mosquitoes - was over expressed at 18 and 24h PBM in *SCD1* KD mosquitoes. Since *SCD1* KD alters the SFA and MUFA ratio in cell membranes by decreasing MUFA, it is possible that *GSTD7* was upregulated as a way to hinder lipid peroxidation in order to maintain homeostasis in *SCD1* KD mosquitoes.

Insect's defense system consists of two main phases: recognition and responses, which are linked by signalling pathways and regulated by modulating elements (Christophides et al. 2004). Pattern recognition receptors such as TEPs and LRIMs recognize PAMP and/ or DAMP and often trigger serine protease cascade (CLIPs) which magnify the danger signals and activate downstream effector processes such as coagulation (MLs, FREPs, Collagens), synthesis of antimicrobial peptides (CECs, GEMs) and melanisation (PPO) (Moreno-García et al. 2014). The mosquito gut is a complex ecosystem, hosting tens of different bacterial genera, which proliferate dramatically after a blood meal. Breakages in the peritrophic matrix and the gut epithelium barrier (Chapter 3) would result in pattern recognition receptors gaining access to bacterial inducers, which would, therefore, lead to over-activation of the immune system (Yassine & Osta 2010). In a manner consistent with this hypothesis, upregulated responses of *CLIPs*, elements of coagulation cascades - *FREPs* and collagen alpha1(IV), complexes of the melanization cascade, such as PPO3, and microbial peptides such as *CEC-1* were observed in *SCD1* KD *A. coluzzii*. However, steady upregulation of the *CEC-1* gene at 0 and 24h PBM in antibiotic-treated *SCD1* KD mosquitoes weakens this argument and indicates activations of immune pathways in response to danger molecules (DAMPs) released by injured or metabolically stressed cells. In mosquitoes, parasite

invasion compromises epithelial cells of plasma membrane and therefore release DAMPs which when recognised by the innate immune system trigger an inflammatory response (Zieler & Dvorak 2000).

Alike vertebrates, invertebrates develop inflammation in response to insults or lesions to cells and tissues which promote immune gene expressions. Wound repair, repopulation of damaged cells and phagocytosis of dead cells are the consequence of inflammations as a part of defense of the body (Moreno-Garcia et al. 2014). However, mechanisms of DAMP pathway activation in *Anopheles* mosquitoes are not clear till to date.

Suppression of *SCD1* - a rate-limiting enzyme that controls the homeostasis of MUFA and SFAs - increases SFA/MUFA ratios and also accumulate ATPs and ADPs in the cell (Scaglia, Jeffrey W. Chisholm, et al. 2009). There is no evidence available for insects that SFA triggers inflammation in mosquito system, however, It has been recognized that, SFAs modulate cellular inflammation by serving as ligands for immune receptors at the cell surface, such as members of the Toll receptor family, in different cells of *SCD1* deficient mice (X. Liu et al. 2011). Several studies have demonstrated that, SFA increases the expression of a number of inflammatory genes by a process that involves (TLR4 - a receptor that binds to bacterial liposaccharide in different cells, such as adipocytes and macrophages (Chait & Kim 2010). Furthermore, genetic and pharmacologic studies have revealed that, fibrinogen induces specific inflammatory functions through cell-specific integrin and non-integrin receptors in different cells types. Moreover, accumulation of stearic acid (an SFA) in the blood plasma induces an increased concentration of fibrinogen, which eventually triggers an inflammatory mechanism in obese people (Ramírez Alvarado et al., 2010). Therefore, upregulation of *FBRPs* may point to an abnormal and excessive saturated fatty acids accumulation, owing to *SCD1* KD and the activation of apoptotic cascades, which are known to share the same approach as the immune pathway.

In brief, transcriptome and metabolome data from *SCD1* KD mosquitoes suggests a global effect of *SCD1* KD mosquitoes on gene expression and metabolites, leading to striking phenotypes such as short life-span, incomplete blood metabolism, and underdeveloped eggs.

Chapter 5

Evaluating effects of *SCD1* inhibitor on mosquito

Summary

Silencing of the stearoyl-CoA desaturase gene *SCD1* in *A. coluzzii* induces dramatic effects including a metabolic syndrome associated with alteration of the lipid contents and other metabolites and prominent immune responses resembling auto-inflammatory reactions. These mosquitoes exhibit significant mortality rates particularly after blood feeding, accompanied by blood leakage into the thorax, inhibition of egg development and interrupted blood digestion, which are incompatible with survival and reproduction. These findings prompted the investigation of *SCD1* as a potential target of small molecule inhibitors *SCD1*, which is described in this Chapter. Sterculic oil (SO) is well-known natural inhibitor of *SCD1*. It inhibits oleate formation through desaturation of exogenous stearate in prokaryotic and eukaryotic cells, and cholesterol biosynthesis. It also alters the permeability of the cell membrane and cell division. An active component of SO is sterculic acid (SA), a potent inhibitor of delta (9) desaturation in various aliphatic acids. Addition of 1mM SA in a human blood meal caused significantly mortality (50%) of *A. coluzzii* females between 20 and 40 h post blood meal (PBM). This observed phenotype was consistent with metabolite data showing a significant reduction of desaturase indices (16:1/16:0; 18:1/18:0) as well as accumulation of various intermediates of the TCA cycle and amino acids, similar to what it was observed in *SCD1* KD mosquitoes as described in a previous Chapter. These data validate the usefulness of small molecule inhibitors of *SCD1* as a tool to understand lipid metabolism in insects.

Introduction

SCD1 is a major rate-limiting, iron-containing fatty acyl desaturase enzyme involved in many biological processes, such as preservation of cell membrane fluidity and signal transduction via the conversion of SFAs (stearic and palmitic acid) to MUFAs (oleic and palmitoleic acid, respectively). It may be therefore exploited as a potential molecular therapeutic target for the treatment of metabolic syndromes such as obesity, type 2 diabetes and various cancers in humans (Malik 2004). Recent evidence has shown that genetic or pharmacologic inhibition of *SCD1* effectively improves metabolic symptoms, such as abdominal obesity, insulin resistance and hypertriglyceridemia, by altering desaturase indices and metabolically activating or inactivating the metabolic pool in preclinical rodent models (Brown & Rudel 2010). Recently, a series of potent and organ-specific *SCD1* inhibitor biomolecules have been used to study the regulation of the cellular metabolism and signalling pathways through the *SCD1* activity (Scaglia, Jeffrey W Chisholm, et al. 2009).

Sterculic oil (SO) is the most recognized natural inhibitor of stearoyl coA desaturase. It inhibits oleate formation (18:1) through the desaturation of exogenous stearate (18:0) in prokaryotic and eukaryotic cell lines, and cholesterol biosynthesis (Zoeller & Wood, 1985; Wältermann & Steinbüchel, 2000). It also causes alterations in the permeability of the cell membrane and cell division (Lam N Nguyen et al. 2014). SO, usually extracted from plant sources such as the seeds of the wild almond tree *Sterculia foetida* and the seeds of the common cotton plant *Gossypium hirsutum*, contains two cyclopropenoic fatty acids, sterculic acid (SA, 55%) and malvalic acid (10%) (Ortinou et al. 2012). SA and malvalic acids are also potent inhibitors of delta (9) desaturation in various C-12 to C-20 aliphatic acids (Hernando et al. 2002). Sterculic acid was first discovered by Nunn in 1952 from *sterculia foetida* oil and named by Schlenk. The chemical names of sterculic acid and malvalic acids are 9, 10-methylene-9-octadecenoic acid and 7-(2-octyl-1-cyclopropenyl) heptanoic acid, respectively (Fig. 5.1). Both *in vitro* and *in vivo* studies have confirmed that the fat composition-manipulating ability of SO is due to a reduction of *SCD1* activity (Ortinou et al. 2012). However, the exact mechanism of this inhibition of the *SCD1* enzyme by SA is still not clear (Wältermann & Steinbüchel 2000).

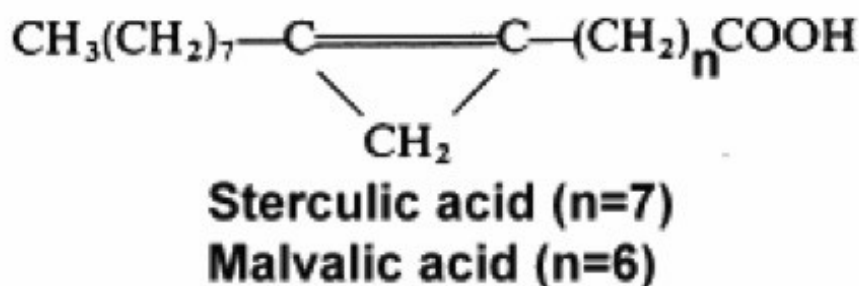


Fig.5. 1 Biochemical structures for Sterculic acid and its lower homologue Malvalic acid

Sterculic acid has 17 carbon atoms and Malvalic acid has 16 carbon atoms. These monounsaturated fatty acids are composed of a 9,10-cyclopropenyl group. (VERMA et al. 1955).

Recent studies support the use of SCDs as potential chemotherapeutic targets for the treatment of various metabolic disorders and infectious diseases (Igal 2011). In the development of a new drug, it is crucial to understand the kinetics of active metabolite formation, in order to predict the therapeutic outcome and to explain the toxicity of specific drugs (Nixon et al. 1977). It has been shown that, labelled SA is absorbed and metabolized at a faster rate in the Wistar rat model when administered by intragastric intubation rather than intraperitoneal injection. The concentration of labelled SA reaches maximum levels in blood serum 2 h after intubation and then drastically drops. At 4 h after intubation, SA accumulates in different organs, with the concentration in the liver peaking at the maximum administered doses. SA is excreted mainly in the faecal matter (48% in urine and 11% in faeces), with a low recovery of labels in the excreted CO₂ at 16h post-intubation (Nixon et al. 1977). The distribution of radioactivity from sterculic acid was investigated in trout, *Salmo gairdneri*. By 168 h, 50% of the administered dose was excreted in feces. Moreover, less than 1% of the dose was released as carbon dioxide during the same time period. At 119 h incorporation of radioactivity into most organs was peaked, and the majority of the label in the liver was in the fatty acid portion of the lipid fraction (Eisele et al. 1979). They observed SA activity in most of the organs in trout.

RNAi are widely used and powerful tool to study gene function by destructing mRNA molecules. However, the use of RNAi is accompanied by several hurdles such as off target activity. Off-target activity can complicate the interpretation of phenotypic effects in gene-silencing experiments and can potentially lead to unwanted toxicity. On the other hands; small inhibitor molecules inhibit protein activity in the cell. Therefore, these two tools can be used in a complimentary manner to screen out offtarget effects.

The metabolic consequences of SA consumption in insects, especially in mosquitoes, are yet to be determined. A few studies, with a range of experimental paradigms, have reported both beneficial and possibly lethal outcomes when SA is consumed. The primary aims of the experiments described in this Chapter were to: (1) examine whether SA that naturally acts against SCD1 can have the same effect on mosquitoes as *SCD1* KD, thus validating the phenotypes reported in Chapter 3, and (2) derive a deeper understanding of the metabolic changes associated with SCD1 inhibition, by assessing the SA effects on the mosquito metabolome and compare them with this of the *SCD1* KD.

Methods

Preparation of SA solution

SA was synthesized by Baird's lab in Bangor University, UK. SA solution was prepared using 1M Tris buffer with pH 8.5. Initially, 1M SA stock solution was prepared, dissolving SA in 1M Tris buffer and kept at -20° C. For experiments, SA stock solution was diluted into tris buffer to get final concentration of 1mM. This diluted SA was mixed with human blood, just before membrane feeding.

Adult drug susceptibility test

Four-day old, sugar fed female *A. coluzzii* mosquitoes were fed on human blood supplemented with 1mM SA in 1mM Tris buffer or with 1mM Tris buffer alone. Mosquitoes were maintained on 5% sucrose solution throughout the experiments for both the drug susceptibility test and the metabolic profiling. Dead mosquitoes from each group were counted every 6 h post blood meal (PMB). Before performing experiments with 1mM SA concentration, different concentration of SA including 0.4, 0.6, 0.8, 1, 1.4, 1.6 and 1.8 mM were tested to determine the IC₅₀.

Metabolomic Profiling

Four to five-day old, sugar fed female *A. coluzzii* mosquitoes were fed on human blood supplemented with 1mM SA in 1mM Tris buffer (experimental) or with 1mM Tris buffer alone (control). Fully-fed female mosquitoes from both the experimental and control groups were sampled at 0 (just prior to blood feeding), 18 and 36 h PBM. Mosquitoes were maintained on 5% sucrose solution throughout the experiment. Samples were prepared according to the procedures described in Chapter 4.

Mosquito fecundity

Female mosquitoes were allowed to feed on 7-8 week old CD1 laboratory mice that were treated orally with SA (1mM) using a feeding needle. Blood-fed mosquitoes were separated from the non-fed mosquitoes and placed into single paper cup. 5 cm filter paper strip and 50ml of salty water was provided inside the cup to allow mosquitoes to lay eggs on it. The number of laid eggs were analysed using the t-test.

Results

SA administration through blood meal causes adult mosquito mortality

A. coluzzii mosquitoes fed on human blood supplemented with 1 mM SA (in 1 mM Tris buffer final concentration) exhibited 52-63% mortality at 40-72 h PBM. Kaplan-Meier statistics revealed that these mortality rates were significantly higher ($p < 0.0001$) compared to control mosquitoes that were fed on human blood supplemented with Tris buffer (Fig.5.2). To investigate the effect of drug-induced SCD1 inhibition on larval survival, SA was added to the larval water at 1 mM final concentration. The control group received Tris buffer, the chosen solvent of SA, at 1 mM concentration.

This data is consistent with those obtained with *SCD1* KD mosquitoes described in Chapter 3. Similarly, excessive chick embryo mortality was observed when treated with 50 mg SO or 25 mg of purified SA (Austic et al. 1971). In addition to experiments in chick embryos, a number of studies have reported attenuated cell proliferation and programmed cell death, or apoptosis, due to aberrant or drug-inhibited expression of the SCD1, in cancer cell lines through a mechanism stimulating the Endoplasmic reticulum (ER) stress signalling pathway (Leung & Kim, 2013; von Roemeling et al., 2013). For example, PluriSIn #1, a cell-specific inhibitor of *SCD1*, is shown to cause death of human pluripotent stem cells and hinder embryonic development by inducing ER stress, protein synthesis attenuation, and apoptosis (Ben-David et al. 2013).

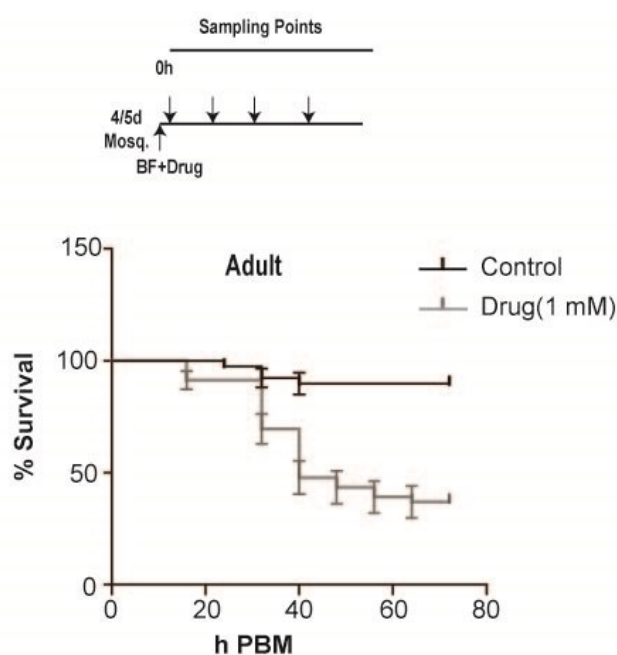


Fig. 5.2 A. *coluzzii* survival after treatment with 1 mM SA

Survival analysis of 4 to 5 day old female *A. coluzzii* with SA or Tris buffer, added to human blood provided through a membrane feeder, and monitored for 80 h PBM. Statistical analyses were performed using Kaplan-Meier and log-rank statistics and indicated highly significant differences between SA-treated and control mosquitoes ($p < 0.0001$). Reported values are the mean $\pm$ SE of three independent biological experiments and $n = 40$ per group per experiment. Schematic at the top of the figure indicates the experimental design for adult mosquitoes.

SA induces metabolic changes in *A. coluzzii* similar to *SCD1* KD

Analysis of temporal metabolic changes in *A. coluzzii* triggered by SA is critical for understanding the abnormalities in the regulation of biochemical pathways and networks and to confirm that the SA phenotypes are indeed due to SCD1 inhibition. This information can offer valuable insights into the effect of SA administration on mosquito physiology.

Four-day old female mosquitoes were offered a human blood meal supplemented with 1 mM SA, while the control mosquitoes were offered a blood meal supplemented with 1 mM Tris buffer. Sampling of mosquitoes was performed at 0hr BBM, 18hr and 36hr PBM.

SA reduces total desaturase indices in *A. coluzzii*:

The desaturase indices 16:1/16:0 (palmitole to palmitate) and 18:1/18:0 (oleate to stearate) serve as a biomarker for the activity of SCD1 (Chajès et al., 2011; Ortinau et al., 2012). SA supplementation caused a significant reduction in desaturase indices at 36 h PBM, indicating an overall increase of SFA content (palmitate and stearate) compared to control *A. coluzzii* mosquitoes (Fig. 5.3). These data are in agreement with the *SCD1* KD metabolic indices (Chapter 4 of this thesis), albeit less pronounced. They are also in agreement with other studies, correlating decreased *SCD1* activity, due to the administration of cyclopropenoic fatty acids (SA and malvalic acid), with increased SFA content (Attie et al., 2002; Ortinau et al., 2012).

SA causes an aqueous metabolite profile that is similar to that of *SCD1* KD:

In order to assess the effects of SA on mosquito physiology, metabolites were analysed using GC-MS before blood feeding and post blood feeding. The analysis showed that, the levels of most of the extracted amino acids, nucleotides, sugars and compounds involved in the urea and TCA cycles increased in SA-treated blood-fed mosquitoes (Fig. 5.4). Most of the metabolites did not show any difference in concentrations between experimental and control groups at 0 h PBM (just prior to SA-supplemented blood meal). Female mosquitoes depend on sugar meal to generate energy before blood meal and move to blood metabolism for energy required for reproduction. During blood meal metabolism, protein-rich liquid meal converts into a huge amount of amino acids. Consistent with this evidence, small traces of amino acids were observed at 0 h PBM, which increased significantly after blood meal ingestion.

Acetyl CoA is the only fuel molecule to enter the TCA cycle and it can be derived from the break-down of fatty acid molecules in mitochondria, through β -oxidation (Schulz 1991). Excess entry of the acetyl CoA into the TCA cycle, or any of intermediates of the TCA cycle, increases all the other intermediates metabolites (Bu & Mashek, 2010). Indeed, a marked increase in the levels of TCA cycle substrates, such as succinate, fumarate, malate and citrate, was observed at 18 and 36 h PBM in SA treated mosquitoes compared to controls, indicating an over-supply of acetyl CoA and up-regulated of β -oxidation. These higher levels of TCA cycle intermediates in SA treated mosquitoes are consistent with the data obtained from *SCD1* KD mosquitoes (Chapter 4).

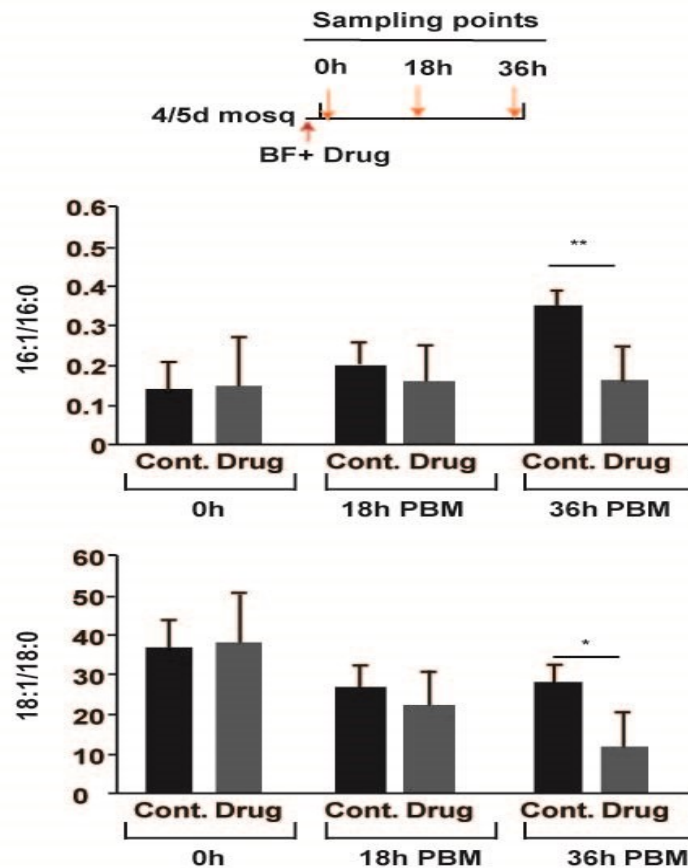


Fig.5. 3 SA supplementation with blood meal reduced total desaturase indices in female *A. coluzzii*

(A) 16:1/16:0 (palmitolate to palmitate) and (B) 18:1/18:0 (oleate to stearate) desaturase indices are shown for control and SA-treated mosquitoes. Reported values are the mean $\pm$ SE of five independent biological experiments and n= 3 per group per experiment. **p<0.001, *p<0.01. Schematic at the top of the figure indicates the experimental design.

The ketogenic amino acid leucine has the highest flux among the principal essential amino acids for lipogenesis in *Ae. aegypti* at 36 h PBM (Zhou et al., 2004a; Zhou et al., 2004b; Zhou & Miesfeld, 2009). In addition to TCA cycle substrates, an increased level of leucine at 18 and 36 h PBM, in SA treated mosquitoes, indicated impaired synthesis of the lipid reserve. These data are in agreement with studies showing that *SCD1* inactivation stimulates metabolic pathways that promote β -oxidation and decreased lipogenesis (Liu et al., 2011; Dobrzyn et al., 2008; Cook & McMaster, 2002).

In consistence with the data obtained from *SCD1* KD mosquitoes, the levels of ornithine, a central component of the urea cycle disposing excess nitrogen in the form of urea, were slightly higher in SA-treated mosquitoes at 18 and 36 h PBM compared to control.

Lower levels of histidine were also observed in SA-treated mosquitoes at 18 and 36 h PBM, also in agreement with the levels of histidine in *SCD1* KD mosquitoes. These data contradict previous findings that level of histidine was significantly higher than all other amino acids after the first gonotrophic cycle (120 h PBM) in *Ae. aegypti* (Zhou & Miesfeld 2009).

Similarly to *SCD1* KD mosquitoes, tyrosine levels were also lower in SA treated mosquitoes at 18 and 36 h PBM compared to the control.

Levels of alanine, proline, serine, glycine, valine, isoleucine and methionine were higher in SA compared to the control group, similar to *SCD1* KD mosquitoes presented in Chapter 4. Alanine, serine, valine and methionine are converted into intermediates of the TCA cycle during amino acid degradation. Therefore, it is likely that accumulation of these metabolites in SA treated mosquitoes is a result of a feedback mechanism due to the build-up of TCA cycle intermediates through over activation of β -oxidation and the TCA cycle.

The profiles of metabolites that were significantly affected by SA treatment compared to controls were also mapped to metabolic pathways and compared with those of *SCD1* KD mosquitoes presented in Chapter 4 (Fig. 5.4). The results from the microarray data described in Chapter 4 are also shown. This analysis highlighted the finding that affected metabolites were mostly related to fatty acid desaturation, β -oxidation, the TCA cycle, the urea cycle and amino acid metabolism. In both SA-treated and *SCD1* KD mosquitoes, the levels of metabolites related to these pathways were higher than those of control mosquitoes.

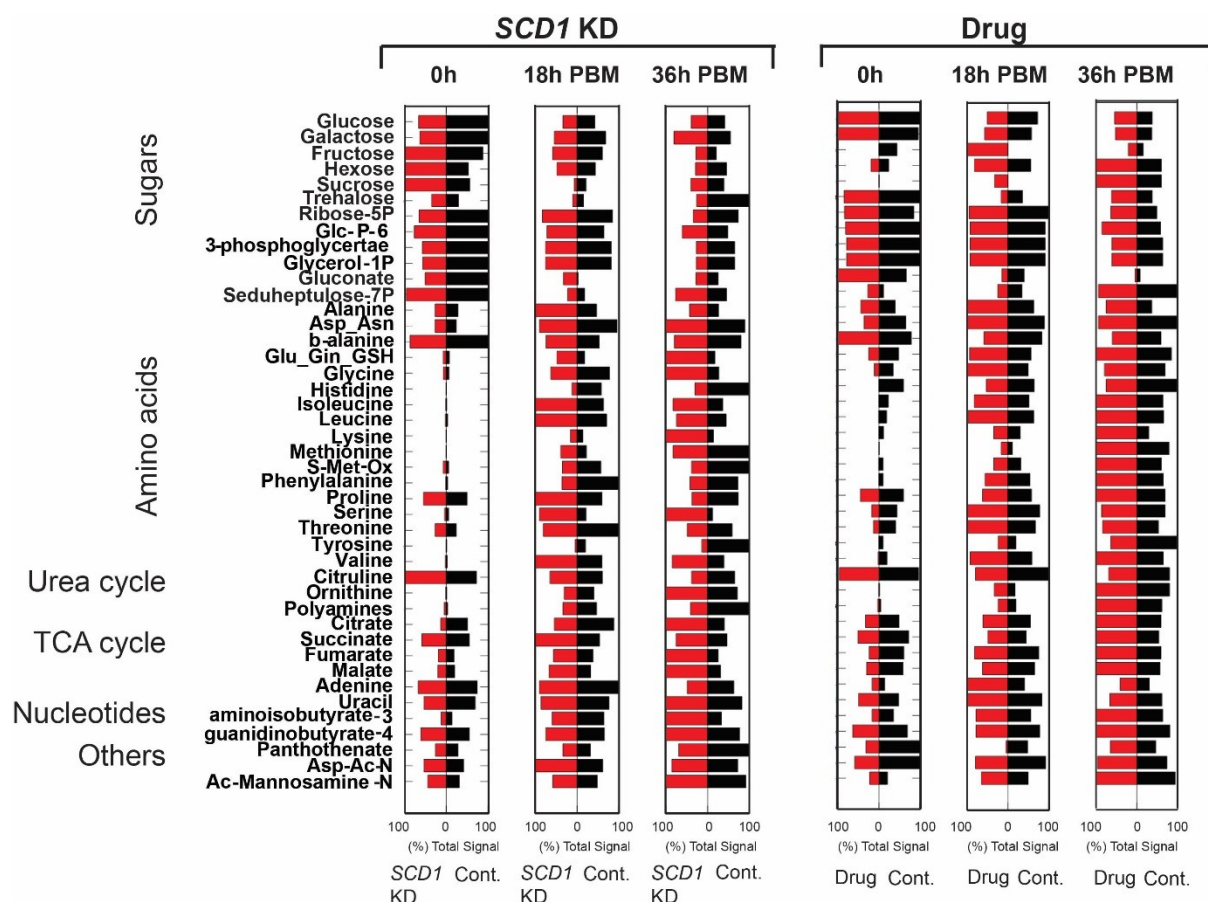


Fig. 5.4 Comparative analysis of *SCD1* KD and SA-treated metabolome

Red bars represent the mean percentage of metabolite signals in *SCD1* KD or drug treated mosquitoes from 5 independent biological repeats. Black bars show mean percentage of metabolite signals in respective controls. Only the metabolites with statistically significant profile changes between experimental and control groups are shown.

Some differences in metabolite levels between *SCD1* KD and SA treated mosquitoes compared to their respective controls were detected, including those related to glycolysis and the pentose phosphate pathway, such as sucrose, 3-phosphoglycerate, glycerol-P, GlcP-6 and ribose 5P. These metabolites remained constant in SA treated mosquitoes but decreased in *SCD1* KD mosquitoes compared to their respective controls. These data suggest that these pathways remain unaffected during the mildest inhibition of *SCD1* by SA treatment compared to the very effective *SCD1* KD, and are consistent with a previous study showing that the effects of SA consumption on glucose metabolism of lean mice are benign (Laura, 2012).

Reduced *A. coluzzii* fecundity after a blood meal on SA treated mice

To further investigate the potential use of SA as a novel target for vector control interventions, an experiment was designed where mosquitoes were allowed to feed on 7-8 week old CD1 laboratory mice that were treated orally with SA using a feeding needle. As presented in Chapter 3, *SCD1* KD *A. coluzzii* exhibited small and undeveloped ovaries following blood feeding. Therefore, this experiment was designed to test whether feeding on SA-treated mice would compromise mosquito fecundity due to inhibition of SCD1.

The dose of SA was calculated so that each mouse received a 1 mM final concentration of SA in the blood. Control mice were treated with Tris buffer at 1 mM final concentration. Each mouse was used to feed three different groups of mated females at 2, 4 and 6 h after oral administration of SA or Tris. The time points were chosen on the basis of reported data on SA metabolism in rats (Nixon et al. 1977). According to these data, the concentration of SA reaches a maximum in the blood serum of all tissues of wistar rats 2 h after intragastric intubation and then drastically drops. At 4 h after intubation, SA accumulates in different organs, with the liver peaking at the maximum administered dose.

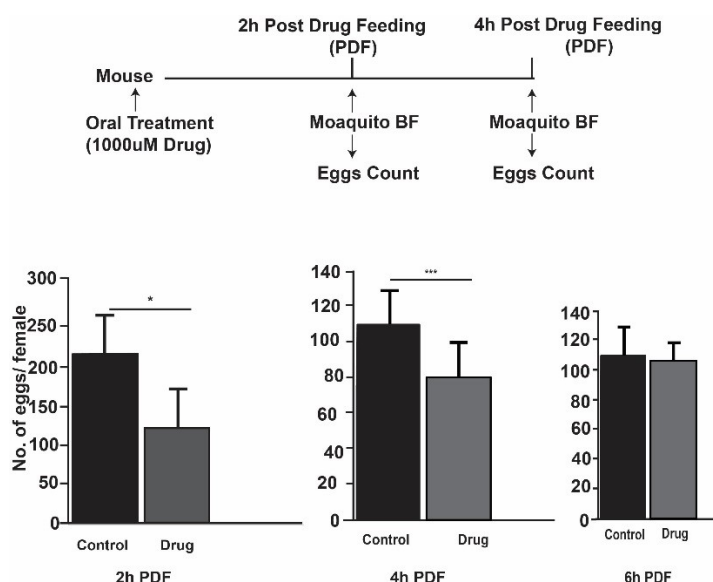


Fig. 5.6 *A. coluzzii* fecundity after blood feeding on SA-treated mice

Mean number $\pm$ SEM of eggs laid by a female *A. coluzzii* mosquito at 2 h (A), 4 h (B) and 6 h post drug feeding (PDF). * $p<0.01$, *** $p<0.0001$. Schematic at the top of the figure indicates the experimental design.

After blood feeding, fully-fed mosquitoes were placed into paper cups that had a wet, 5 cm, filter paper strip. The number of eggs laid per female was analysed using a t-test. Three independent biological replicas were performed. The results showed that mosquitoes that fed on an SA-treated mouse laid a markedly reduced number of eggs (Fig. 5.6). The mice used for the experiments were kept in the cage till the end of experiment. After that mice were culled according to health and safety rules. No significant changes in mice movement were noticed during this time period.

Mosquitoes that fed on a SA-treated mouse at 2 h post drug feeding (PDF) laid 38% less eggs than mosquitoes that fed on a control mouse ($p < 0.01$). A 28% egg reduction was recorded with mosquitoes that fed on the same mouse 2 h later (4 h PDF; $p < 0.0001$). No differences in egg numbers were observed at 6 h PDF between experimental and control mosquitoes. These data suggest that SA treatment of the vertebrate blood source inhibits the activity of mosquito SCD1 thus compromising ovarian development. They also indicate that SA levels in the blood stream decline rapidly after administration, reaching levels that are insufficient to inhibit mosquito SCD1 activity.

Discussion

The induction of lipotoxicity by excess SFA, in an SCD1 inhibited cancer cell model, leads to cell dysfunction and mortality, by increasing basal apoptosis (Scaglia et al. 2009). Previous studies have demonstrated that, excessive accumulation of palmitic acid (i.e. palmitate), the most common SFAs in animals, induces apoptotic cell death by inducing ER stress (Guet al., 2010; Park et al., 2014; Zhang et al., 2012). The results described in this chapter reveal significant mortality of *A. coluzzii* mosquitoes 20 and 40 h after feeding on human blood supplemented with 1 mM SA. This phenotype concurs with a significant reduction of desaturase indices 16:1/16:0 (palmitoleate to palmitate) and 18:1/18:0 (oleate to stearate) detected at 18 and 36 h post blood feeding. However, the reduction of total desaturase indices after SA treatment is less pronounced than that in *SCD1* KD mosquitoes.

Since the reduction of desaturase indicates accumulation of excess SFA in cells (Matsui et al. 2012) due to SCD1 inhibition (see also Chapter 4), it is likely that lipotoxicity is a major cause of death following SA treatment. These data are in agreement with previously reported data, where loss of SCD1 activity leads to significant accumulation of SFAs and reduced viability of *Plasmodium* parasites and chick embryo mortality (Gratraud et al., 2009; Austic, Hill, & Wilson, 1971).

Embryo mortality was observed by several studies, in which 50 mg of SO or 25mg of purified SA were administered daily to individual hens (Austic et al., 1971). A number of additional

studies have reported attenuated cell proliferation and programmed cell death due to aberrant, or inhibited, expression of the *SCD1* gene in cancer cell lines, triggered by a stimulation of the ER stress signalling pathway (Leung & Kim, 2013; von Roemeling et al., 2013). Similarly, PluriSIn #1, a cell specific inhibitor of *SCD1*, leads to death of human pluripotent stem cells and hinders embryonic development, by inducing ER stress, protein synthesis attenuation and apoptosis (Ben-David et al., 2013). Significant mortality has been also observed at 20 h PBM, in SA-treated blood-fed *Ae. albopictus* mosquitoes that are major vectors of the dengue virus.

It has been demonstrated in different animal models that *SCD1* inhibition leads to accumulation of SAFs suppressing *ACC* through a well-known feedback mechanism, resulting in the drop of malanoyl-CoA concentration (Palton, 2007). Malonyl-CoA derepresses carnitine-palmitoyl-transferase (CPT), which in turn increases the transport of acetyl-CoA into the mitochondrial β -oxidation. The excess entry of acetyl-coA into the TCA cycle, or any of the intermediates of the TCA cycle, increases all other intermediates, as one is converted into the other (Stryer, 1995; Bu & Mashek, 2010). Consistent with these findings, in the current study, inhibition of *SCD1* in *A. coluzzii* mosquitoes by *dsSCD1* or SA treatment has also led to accumulation of various intermediates of the TCA cycle, such as citrate, succinate, fumarate and malate, at 36 h PBM.

Likewise, a higher level of methionine at 36h PBM, in both SA treated mosquitoes and *SCD1* KD mosquitoes as compared to control mosquitoes, was noticed. Methionine plays a key role in the synthesis of the protein carnitine, which helps long, branched-chain fatty acid molecules to pass through the inner mitochondrial membrane. This indicates an up-regulation of CPT (which encodes for the carnitine protein) which may consequently increase mitochondrial β -oxidation, in blood-fed, SA-treated mosquitoes. Previous studies demonstrate that methionine reduces fat deposition in the liver by dissolving fat (Svegliati-Baroni et al., 2006).

The levels of metabolites which are closely related to the TCA cycle, such as alanine, proline, serine, glycine, valine and methionine, were higher in both *SCD1* KD and SA treated mosquitoes, compared to the controls. Therefore, it is likely that, the accumulation of these metabolites happened via an efflux of intermediates, and an over-activated TCA cycle, in *SCD1* KD and SA treated mosquitoes. It also suggests that, *SCD1* inhibition affects amino acids flux in mosquitoes, since serine is the precursor of glycine, proline can be biosynthetically derived from glutamate and alanine can be synthesized from valine, leucine and isoleucine. This accumulation may increase cellular toxicity, leading to metabolic

syndromes in SA treated and *SCD1* KD mosquitoes, since some amino acids, like proline, have been considered to be a potential endogenous excitotoxin in humans.

On the other hand, the levels of a few amino acids, like histidine and tyrosine, were lower in both *SCD1* KD and SA treated mosquitoes, compared to control mosquitoes. It has been reported that, strong increases in SFA cause alter metabolic fluxes in TCA cycle which start ROS accumulation in palmitate treated hepatic cells (Leamy et al. 2013). Less accumulation of Histidine was observed in SA fed female mosquitoes, at 18hr and 36hr PBM, compared to the controls. Hence, elevated levels of TCA cycle substrates in SA treated mosquitoes, where SFA accumulation was significantly higher than that of the controls, suggest the possible induction of oxidative stress in the cells (no data available). It has been shown that, H₂O₂-stressed cells contain a significantly higher level of succinate and citrate, and a lower level of histidine, which possibly induces ketoglutarate accumulation, for the purpose of combating oxidative stress (Lemire et al., 2010). Therefore, the elevated TCA cycle fluxes (higher levels of citrate and succinate) and lower levels of histidine, in the SA administered mosquitoes at 18 and 36h PBM, may be pointing towards metabolic networks which operative, in the mosquito system, as an antioxidative defense. However, histidine was incorporated into the amino acid pool and excreted at its highest level after the first gonotrophic cycle (120h PBM) in *Ae. aegypti* (Zhou & Miesfeld, 2009).

Interestingly, the glucose metabolism was affected in *SCD1* KD mosquitoes, whereas in SA treated mosquitoes, it remained unaffected. Most of the metabolites related to glycolysis or the pentose phosphate pathway, such as sugar, 3-phosphoglycerate, glycerol-P, GlcP-6 and ribose 5P, remained constant in SA treated mosquitoes, although these metabolites decreased in *SCD1* KD mosquitoes. This data shows consistency with a previous study, where the effects of sterculic oil consumption, in lean mice, appeared to be benign, as glucose metabolism was not altered (Laura, 2012). It has been demonstrated that, synthesis and release of trehalose is coupled to fatty acid oxidation in two different species of cockroach. In agreement with this study, the levels of trehalose were higher in SA treated mosquitoes. However, the release of trehalose was lower in *SCD1* KD mosquitoes, compared to that of the control mosquitoes. It is possible that trehalose synthesis was unaffected in SA treated mosquitoes, since the glucose metabolism was unaltered.

Additionally, higher levels of phenylalanine were noticed in SA treated mosquitoes, compared to the controls, at 36h PBM, in contrast with phenylalanine levels in *SCD1* KD mosquitoes. Surprisingly, the levels of tyrosine, which was synthesized from phenylalanine by the activity of Phenylalanine hydroxylase (PAH) enzyme, were lower than the controls, at 18h PBM and 36h PBM. Tyrosine plays an important role in egg formation by synthesizing

yolk protein. The inhibition of PAH, leading to low levels of tyrosine, causes small sized ovaries in blood-fed, female *A. gambiae* (Fuchs et al., 2014). Therefore, these altered levels of tyrosine may correspond to the phenotype of significantly less numbers of eggs being laid by mosquitoes, which were fed on mice treated with SA, at 2h PDF and 4h PDF. The accumulation of phenylalanine and the low levels of tyrosine may suggest that, SA treated mosquitoes may have an altered conversion pathway between phenylalanine and tyrosine.

Mated females of *A. coluzzii*, which were fed on an SA treated mouse, both at 2h and 4h PDF, laid a markedly reduced numbers of eggs (a 38% reduction in egg numbers among mosquitoes fed at 2h on a PDF mouse and a 28% reduction in egg numbers among mosquitoes fed at 2h on a PDF mouse). This is the first time, to date, that mosquitoes were fed on an SA treated mouse, in order to examine the effect of digested SA on mosquito fecundity, after blood feeding. However, several studies have demonstrated that, cotton seed meal, which contains sterculic acid, or SA supplementation with food cause defective fecundity in animals. For instance, dietary SA and malvalic acid produce sterile females, suppress egg production, reduce pupation and lead to the emergence of unhealthy pupa in the blow fly and face fly (Binder & Chan, 1982). In addition to insects laying defective eggs, discoloured egg albumin and a weakened vitelline membrane were also noticed in hens, fed on cottonseed meal (Deutschman et al., 1961). Unfortunately, there is no data available for the fate of the eggs laid by SA fed mosquitoes, in this particular project. It would be very exciting if, the development and growth of larvae, pupae and adults from these eggs could be monitored in future studies.

Chapter 6

Climate change and human vulnerability to VBDs in developing countries: country profile of Bangladesh

Summary

Vector-borne diseases are a major public health concern in Bangladesh. This chapter aims to provide an epidemiological overview of the most important of these diseases including Malaria, Visceral Leishmaniasis (VL), Dengue Fever (DF), Chikungunya Fever (CHIK), Lymphatic Filariasis (LF) and Japanese Encephalitis (JE). The information was accumulated from relevant literature indexed in PubMed from 2000 to 2014 and from local journals with Medical subject headings. The mortality and morbidity rates are highest for malaria among all other vector-borne diseases, followed by VL. However, recent changes in population dynamics and living standards in terms of urbanization, migration and growing urban slums have drastically increased the possibility for outbreaks of urban diseases such as DF. This together with recent reports of drug resistant pathogens, insecticide resistant vector populations and outbreaks of neglected diseases in neighbouring countries including India and Myanmar cause an alarming situation and stress the need for a national strategy to deal with potential significant outbreaks in the future.

¹In the chapter 6 of this thesis, the author reviewed the current situation of vectorborne diseases in Bangladesh as a part of the requirements from her scholarship body (Commonwealth Scholarship commission)

Introduction

Climate change - a complex system of environmental changes occurring around the world with unknown future risks to humans and natural ecosystems, has a significant effect on vector-borne diseases. In light of this issue, developing countries are at high risk of public health disasters due to a combination of rapid population growth, urbanization, economic instability and chemical pollution. This review of epidemics of major VBDs in Bangladesh focuses on the current status of the VBDs and also provides information on research gaps to be identified in future.

Bangladesh, a South Asian developing country, scores top position in the German Watch's Global Climate Risk Index (CRI) of 2011, owing to its position as the state most vulnerable to global climate change in coming decades. Along with climatic vulnerability, other factors, such as geographic and regional location, high human population growth, poverty, land use, deforestation, urbanization, chemical pollution, insecticide and drug resistance, changes in public health policy, trade, travel and genetic changes in pathogens, are increasing the probability of a public health disaster such as the sudden outbreak of VBDs in this country. In essence, it is necessary to understand the recent VBD epidemiology, identify all present hurdles to address VBD-related public health issues and future opportunities and to adjust the priorities of the country in all possible spheres. Current knowledge on the prevalence, distribution, and disease burden of the major vector borne diseases in Bangladesh has been summarized in this part of the chapter.

Geography of Bangladesh

Bangladesh is the world eighth-most populous country with over 160 million people (Bangladesh Bureau of Statistics 2013). It is divided into 7 major divisions that are subdivided into 64 administrative districts (Fig.7). It covers an area of 147,570 km² and is mainly low-lying delta plains with few hilly and largely forested regions in the east and northeast that are about 8% of the land. The country shares 4,053 km borders with India (29 districts) and 193 km borders with Myanmar (3 districts).

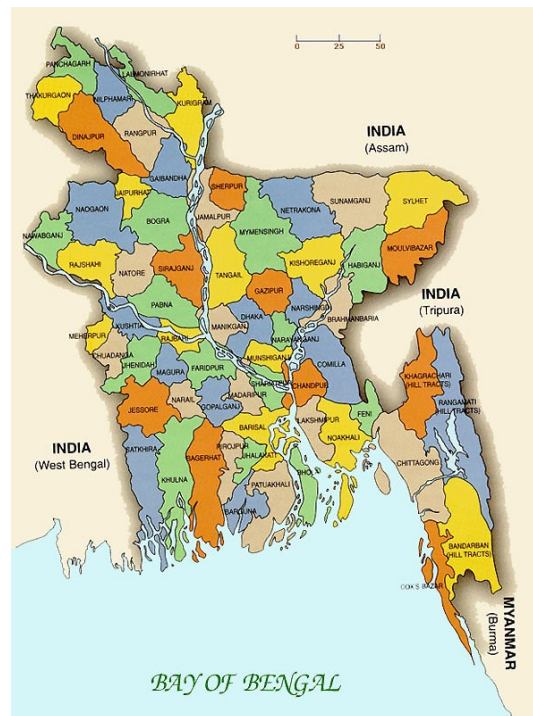


Fig 6.1 Administrative map of Bangladesh

Bangladesh is divided into 7 major divisions and sub-divided into 64 administrative districts with other sub-units. Bangladesh shares border with India and Myanmar. Small hilly regions in the North – East and South–East, with an average elevation between 244 and 610 meters. Hilly and forested areas (8% of the total land) cover the Eastern and North-Eastern border region of the country. Source: www.worldmap.co

There are 4 main seasons: winter (December-February), pre-monsoon (March-May), monsoon (June-August), and post-monsoon (September-November). Monsoon is characterized by heavy seasonal rainfall of 1,500-2,500 mm, with a maximum of 3750 mm at the eastern borders, while the winter is short and dry (Shahid 2012). Temperatures range from 30°C to 40°C and are higher in inland than on the coast. In most parts of the country, April is the hottest month and January is the coolest month of the year. The average relative humidity throughout the year ranges between 45% and 92%. Regional climatic variations are minor (Varmus et al. 2003).

The burden of vector-borne diseases is very high in Bangladesh due to its geographic location, high human population growth, poverty, climate, excessive land use and deforestation, urbanization, insecticide and drug resistance, lack of consistency in public health policy and trade (Haque et al. 2012; N. Islam et al. 2013; Ahmed et al. 2013). Malaria, VL, DF, CHIK, LF and JE are currently the most frequently reported cases. Here, we

summarize current knowledge on the prevalence, distribution and burden of these diseases in Bangladesh. Such reviews are critical for designing effective integrated vector and disease management programs.

Methods

The data were collected from existing literature deposited in PubMed between 2000 and 2014 and from local journals with Medical Subject Headings, and were synthesized before presentation. Geographic Information System (GIS) shape files were obtained from the Bangladesh Agricultural Research Council (BARC), and spatial disease distribution was represented graphically using the ArcGIS 10.2 software.

Results

Malaria

Endemicity:

Bangladesh is one of the ten South-East Asian countries where Malaria is endemic. More than 16 million people, which is the 10% of the total population, live in these malaria endemic areas (Haque et al. 2009). In 2000- 2011, the number of reported malaria cases in Bangladesh were 1.254 million. The number of malaria cases each year was close to stable (~54,000) from 2000 to 2011 with a significant increase in 2008 (84,690) (N. Islam et al. 2013). In recent years, malaria has become endemic (95-98% of malaria cases) in 13 Eastern and South-Eastern districts. Among these 13 malaria endemic districts are 8 North-Eastern districts - Kurigram, Sherpur, Mymensingh, Netrokona, Sunamganj, Sylhet, and Habiganj which share a border with India. Included are also 3 South - Eastern districts - Khagrachhari, Rangamati and Bandarban which are collectively known as Chittagong Hill Tracts (CHT) and share borders with India and Myanmar as well as two other districts are on the South-West of the country (Chittagong and Cox- Bazar) (Elahi et al 2012; Chowdhury et al. 2010; Paul.1984). In the past few decades, malaria distribution has changed significantly with some new districts emerging as malaria endemic, namely: Kurigram, Sherpur, Netrokona, Sunamganj, Habiganj, Maulavibazar; while, simultaneously, many other districts have become malaria-free. A national Survey from 1968 to 1977 reported malaria incidents in Dhaka, Tangail, Faridpur, Noakhali, Comilla, Rajshahi, Pabna, Bogra, Rangpur, Dinajpur, Jessore, Kushtia, Barisal, Patuakhali, and Khulna - all districts which are no longer considered as malaria risk areas (Bashar 2012) (Fig.6.2A).

Malaria transmission also varies across these districts due to their varied geographic position, rainfall, temperature and level of forestation. A positive correlation between climate

variables such as high rainfall, high temperature, and high humidity and malaria cases was reported mainly from CHT districts. Hilly and remote districts like Chittagong, Rangamati, Khagrachari, Bandarban and Cox's bazaar are hyper-endemic with a disease prevalence of 11% or higher. Among these hyper-endemic districts, extremely high prevalence (36%) has been recorded in Bandarban. Other malaria-endemic districts are prone to low-level endemicity with an overall prevalence varying from 3.10-3.97% (Haque et al. 2011).

Recent studies have identified malaria hot spots in the Khagrachari and Rangamati districts. In Khagrachari, five malaria clusters were identified – with one being a most-likely cluster and four secondary clusters (Elahi 2012). The highest prevalence (22%) was reported in Dighinala (one of the subunits of this district), which has common borders with India and Myanmar. A single most-likely cluster and two secondary clusters were also recognised in Rangamati (Haque et al. 2009).

Diagnosis:

Several diagnostic methodologies have been used to identify symptomatic malaria cases in Bangladesh. These include microscopic examination, Rapid Diagnostic Tests (RDT), Nested Polymerase Chain Reactions (PCR), Immuno-Chromatographic Tests (ICT) and dipstick antigen capture assays (Para SightTM-F test). RDT was introduced in the country in 2006 as the part of the national malaria control program funded by the Global Fund for AIDS, TB and Malaria (GFATM) (Elahi et al. 2013; Alam et al. 2011; Jahan et al. 2011; Fuehrer et al. 2011). As a result, blood slide analysis is no longer used as the main malarial identification tool in Bangladesh, but rather it only used to support early diagnosis and rapid treatment.

Seasonality:

Malaria transmission in Bangladesh is mostly seasonal and limited to the regions bordering Myanmar in the East and India in the North. During the cool and dry season, mosquitoes were relatively less active and the number of malaria cases was relatively small with malaria cases increasing considerably during warm and wet seasons. Malaria peaks during the monsoon in Bangladesh (between May and October) with the highest incidence being in June/July (which may extend up to September) and the lowest being in November (World Health Organisation 2014).

Vectors & insecticide resistance:

The latest check-list of mosquitoes in Bangladesh was developed in 1987. This checklist confirms the presence of 34 species (spp) of anophelines and 79 species of culicines in Bangladesh. Up until 2009, only 7 spp of these 34 Anophelines were documented as competent malaria vectors (Ahmed 1987; Elias et al. 1082). Among these competent vectors,

7 spp are considered to be primary vectors responsible for the transmission of malaria in most of the hilly, forested and foothill areas, flood plain deltaic regions and coastal areas respectively in Bangladesh throughout the year. The remaining three, of these seven, are considered to be secondary vectors as they were reported only in epidemic outbreak situations.

Currently in abundance, *A. annularis* has become an important vector in some places and *Anopheles vagus* - fairly common all over Bangladesh - has been incriminated as a vector. In terms of abundance and incrimination, along with some existing vectors such as *A. Maculatus* and *A. Nivipes*, a couple of newly reported vectors - *A. jeyporiensis* and *A. kochi* - play important roles in malarial transmission in CHT (Alam et al. 2012). *A. baimai*, *A. minimus* s.l., *A. annularis*, *A. jamesii*, *A. maculatus* s.l., and *A. pallidus* are more or less anthropophilic, whereas most of the other species are zoophilic and, as a result, mosquitoes have recently become more exophagic (Rahman 2013). These exophagic traits may be selected by the insecticidal pressure of bed nets (Bern & Chowdhury 2006).

It is notable that, after intensive vector control initiatives using DDT in the 1950s and 1960s throughout the country, most of the primary vectors such as *A. minimus*, *A. philippinensis*, *A. sundaicus* which were eliminated, have recently reappeared since the 1980s, thereby revealing their resistance to DDT. In addition to the primary vectors, secondary vectors like *A. aconitus*, *A. annularis* and *A. Vagus* have been found to also be DDT-resistant (Maheswary et al. 1992; Maheswary et al. 1993; Maheswary et al. 1994).

Major pathogens:

Plasmodium falciparum is the predominant species in the malaria-endemic areas of Bangladesh, with a countrywide prevalence of 3.58%. Historically, all microscopically identified malarial infections in Bangladesh were attributed either to *P. falciparum* or *P. vivax* (Haque et al. 2009; Faiz et al. 2002; Ahmed et al. 2009). To avoid mis-diagnosis, particularly in cases of mixed infection or low parasitemia (Hänscheid 2003; Payne 1988), Bangladesh has recently started using commercially available RDTs, despite the fact that commercially-available RDTs were found to be quite sensitive and specific to *P. falciparum* detection in comparison to *P. vivax* detection in contrast to real-time PCR assay (Alam 2011). The contributions of less prevalent and less well-documented parasites like *P. malaria* (1 among 6685 patients) and *P. ovale* (1.6% among 189 malaria-positive samples) in malaria cases were first reported in a couple of very recent surveillance studies which were based on molecular tools such as genus- and species-specific nested polymerase chain reactions (Broek et al. 2004; Haque, Huda, et al. 2009; Fuehrer et al. 2014). However, the presence of these two malarial parasites was first confirmed by nested PCR in two

border-sharing countries of Bangladesh, Myanmar and India two decades before (Jambulingam et al. 1989; Win et al. 2002). This indicates either the probability of the presence of these parasites in Bangladeshi malarial patients a long time before they were first reported or to their regular migration through the means of human smuggling and human trafficking from countries neighboring Bangladesh in the recent past which may have introduced these parasites to the Bangladeshi population (Wickramage et al. 2013).

P. falciparum has a known resistance to sulfadoxine / Pyrimethamine and chloroquine and is the dominant parasite in malaria –endemic districts, causing more than 87% malarial cases with a country-wide prevalence of 3.58% (compared with only 0.21% for *P. vivax*). A recent molecular tool-based study in the Chittagong Hill Tracts has reported a high rate of mixed parasite infections, where 81.5% were infected with *P. falciparum* (of which 68.3% were monoinfections), 26.5% with *P. vivax* (of which 15.3% were monoinfections), 3.7% with *P. malariae* and *P. ovale* (variant type) with 1.6%. In symptomatic, malaria-positive patients, infection with *P. ovale wallikeri* was higher than that of *P. ovale curtisi*, although the contributions of these species to malarial infection were nearly the same in malaria-positive asymptomatic participants. No malaria case of *P. knowlesi* has been reported so far in Bangladesh, however, the presence and risk of this parasite cannot be ignored, due to the difficulties in identifying and distinguishing it from *P. malariae* by microscopy and the presence of its definite host (*A. leucosphyrus*) and intermediate host (critically endangered *Macaca fascicularis* confined to South-Eastern areas of the country).

Drugs and drug resistance:

In Bangladesh, combinations of artemether and lumefantrine drugs are nationally used as first-line treatment for *falciparum* malaria. Doxycycline + quinine, and tetracycline + quinine combinations are provided in cases of treatment failure in malaria patients with *P. falciparum*. Artemether and quinine are used for the treatment of severe malaria, while chloroquine -primaquine combination (14-day therapy) is administered for *vivax* malaria.

Malaria parasites in Bangladesh have become resistant to many of these drugs. Recently, the degree of *in vivo* and *in vitro* Chloroquine resistance has become unacceptable across the malaria endemic areas and showing an increase from 10% in 1979 to 45% in 1987 and 57% in 1992. Noedl reported that 84% (37 of 44 malaria patient isolates) parasites (*P. falciparum*) were resistant to Chloroquine and that 61% (27 of 44 isolates) parasites were mefloquine resistant in Chittagong in 1999, despite Mefloquine being effective even in 1993 in the Myanmar- Bangladeshi border area (Fuehrer et al. 2010). Combinations of Pyrimethamine / Sulfadoxine (SP) are also not performing successfully in the treatment of malaria cases. In Chittagong Hill Tracts, the majority of malaria cases were Chloroquine-

associated infection and 17.2% of these infections showed unsuccessful parasite clearance and re-occurrence of the disease within 28 days after treatment. Point mutations (94% K76T) at the Chloroquine resistance transporter gene (Pfcr) and at the Multidrug resistance gene (70% N86Y) were very common in these districts(Fuehrer et al. 2012).

SP resistant genotypes have significantly increased in past few years in the border-sharing malaria endemic areas. Most of the parasites showed two or more mutations at SP resistance marker gene Dihydrofolate reductase gene (dhfr) and at the Dihydropteroate Synthase gene (dhps). Identical, or similar, microsatellite haplotypes flanking dhfr of the quadruple mutants in Bangladesh, Thailand and Cambodia, suggest the migration of the parasites to Bangladesh from Myanmar (Fuehrer et al. 2010).

Since 2012, there has been no indication of artemisinin resistance in Bangladesh, however, a very recent study has confirmed a novel A578S mutation in the artemisinin resistance marker K13 propeller gene, which lies adjacent to the C580Y mutation and is the major mutation observed in Cambodia and is thereby suggesting the presence of artemisinin resistance in certain districts of Bangladesh(Fuehrer et al. 2011; Fuehrer et al. 2012; Fuehrer et al. 2014).

Dengue Fever

Endemicity:

Dengue fever is one of the most important emerging acute mosquito-borne arboviral human diseases. In Bangladesh, both classical Dengue Fever (DF) and Dengue Haemorrhagic Fever (DHF) are present. The first confirmed outbreak was documented in 1964 in Dhaka city, followed by scattered cases of DF during the periods of 1977- 1978 and 1996-1997(Ahasan et al. 2008). Nevertheless, the first DHF outbreak (5555 cases and 93 deaths) started in late June 2000, peaked in September and decreased in the dry Winter season in December, 2000 in Dhaka city(Karim et al. 2012). Since then, DF infection has become endemic in Bangladesh. DF cases were detected throughout six divisions of Bangladesh in a hospital-based study in 2008-2009 and was mostly prevalent in the Dhaka division (21/1000 patients admitted) followed by, the Chittagong division (15/1000) and the Rajshahi division (7/1000)(6.2B).

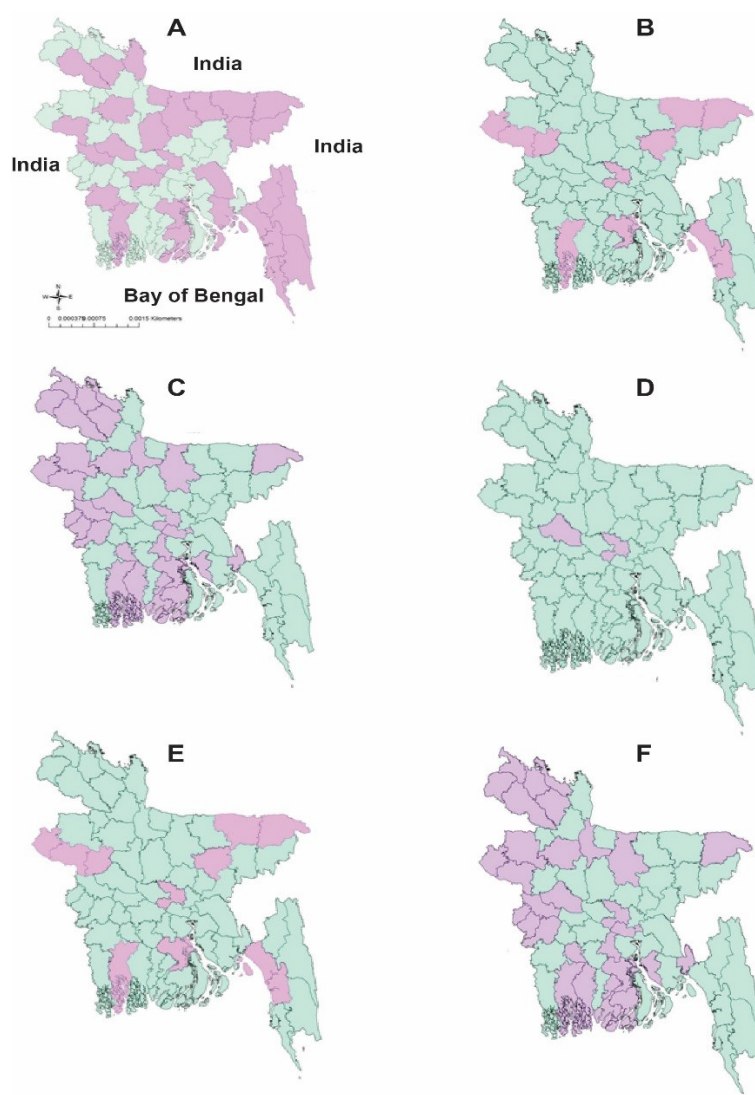


Fig. 6.2 Distribution of major reported vector borne diseases in different districts in Bangladesh

Divisions are shown here using different colours and area of each district is shown by lines. Due to the insufficient available data, district that report at least 1 case throughout the time period of any kind of survey is designated as “case reported” and areas with no case history considered as “No”. Map showing distribution of reported cases of malaria fever from 2000-2011. (A) Malaria was reported from all the major divisions. However, South – Eastern districts are most endemic (B) Distribution of reported cases of dengue fever from 2000-2011. DF was reported from the major 6 divisions – Dhaka, Chittagong, Khulna, Barisal, Sylhet and Rajshahi (C) Distribution of lymphatic filariasis (D) Map showing districts from where Chikungunia infection was reported from 2008-2009. (E) JE infection was reported from different parts of Bangladesh (F) Distribution of Visceral leishmaniasis (VLD) between 1999-2008. The Geographic Information System (GIS) shapefiles were collected from Bangladesh Agricultural Research Council (BARC). The disease dataset was generated from the information extracted from the existing literatures using the online database PubMed from 2000 to 2014 and local journals with the Medical Subject Headings. Finally, the spatial distribution of the diseases was represented graphically (Figure 1 A-F) using ArcGIS 10.2 software.

Less than 1/1000 dengue patients were reported in the Barisal division. According to the Health Ministry of Bangladesh, a total of 23,518 cases and 239 deaths were reported during the period of 2000–11. The highest caseload was recorded in 2002 (6,132 cases), followed by 2000 (3,964 cases), and the lowest caseload was in 2010 (409) (Faruque et al. 2012). DF was primarily reported in two cities, Dhaka (about 92% during the period of 2000-2011) and Khulna. Most dengue cases have been reported in the metropolitan cities of Dhaka, Chittagong, Khulna, and Rajshahi during outbreaks (Fig.9). Geographic Information System (GIS) technology has shown that dengue clusters are less identifiable in areas further away from major hospitals, indicating that the proximity to hospitals determines the diagnosis of dengue (Ali et al. 2003). A large occupational transmission of dengue fever was reported in Dhaka city in 2001, with a 12% attack rate in an occupational setting (Wagatsuma et al. 2004). Adults appear to fall ill with dengue more often than children. Children of 6.5 ± 3.5 years, with an age range of 6 months to 15 years, were the main group affected by dengue infection (without any sex preference; male: female was 1:1) in Dhaka city.

One recent study projected that there will be an increase of 16,030 dengue cases in Dhaka by the end of this century, due to a predicted temperature increase of 3.3°C given a lack of any adaptation measures and changes in socio-economic condition. The major clinical symptoms are fever, anorexia and vomiting, with some headache, and general body aches and a few rashes. DHF patients usually present with ascites, pleural effusion and CNS symptoms (Alam et al. 2010).

Seasonality:

Though Dengue cases have been reported throughout the year, DF season starts in July, peaks in August (37.5% of total annual cases) and subsides in October (Faruque et al. 2012).

Vectors:

So far, very few studies had been done with the purpose of examining the population density of major DF vectors (*Aedes aegypti* and *Aedes albopictus*) in Bangladesh (Chowdhury et al. 2014). These two major vectors were identified during different outbreaks and high numbers of *Ae. aegypti* were identified throughout Dhaka city in the year 2000, though more *Ae. albopictus* larvae than that were found in a stagnant water-covering surface lid in a recreation club in Dhaka city in 2001 (Ali et al. 2003; Wagatsuma et al. 2004).

Pathogens:

The dengue virus is an RNA virus and consists of 4 serotypes - DEN 1 - 4. A study which was conducted during a recent outbreak of dengue suggests that all four serotypes of dengue viruses were circulating nationwide, with a predominance of DEN-3 and occasional co-infection with other types and a minor infection with DEN-1. Virus isolation and serology confirmed the existence of DEN-3 in Dhaka city during the first reported outbreak of dengue in Bangladesh in 1964. Other studies showed the presence of three serotypes, except DEN-1, in Chittagong in the South-Eastern part of the country (Karim et al. 2012). The presence of DEN-1 was reported in several studies conducted in Dhaka city in 1980 and in 1982-1983. Among 2,465 schoolchildren, 278 (11%) were serologically positive against DEN-1 in 1982-1983.

Lymphatic filariasis

Lymphatic filariasis is a dreadful disease in humans caused by the parasitic nematodes *Wuchereria bancrofti*. It afflicts more than 250 million people in tropical countries. The exact figures of filariasis in Bangladesh are not known, however, 34 (out of 64) districts are known to be lymphatic filariasis endemic in Bangladesh, with effects more keenly felt in the Northern districts (Fig.6.2C). 70 million people are estimated to be at risk of this disease, 10 million have various forms of clinical deformity and another 10 million are microfilareemics. Nilphamari, Kisorgonj and Sayedpur Thanas under the Nilphamari district are the most endemic, amongst all other districts, for lymphatic filariasis (Mondal et al. 2011). Studies reported that 1.34% of the total inhabitants of this most endemic district were infected with filariasis, with females being infected significantly more than males and mostly affecting people from poorer classes. Adults (26-45 years) were more affected (53.07%) by this infection than young children 6-15 years (1.92%) (Saha & Mohanta 2011). To stop the transmission of the disease, a two-tablet dose at timed regimens of diethylcarbamazine (DEC; 6 mg/kg) and albendazole (400 mg) or albendazole (400 mg) + ivermectin (200 mcg/kg) was used for a successive 5 years in Bangladesh to eliminate filariasis. Mass Drug Administration (MDA), which started from 2001 targeting one district, has scaled up to 19 endemic districts in 2010. In 2008, 92% of people from two major endemic districts were covered by a 7th round MDA coverage program. Standard blood filming and immune chromatographic techniques (ICT) are commonly used in Bangladesh. Since these tests are not highly sensitive to a low density of parasites, urine-based ELISA method can be very useful to identifying post-MDA low endemic stages in Bangladesh (Hafiz 2013).

Chikungunya Fever

Chikungunya is a fever caused by the chikungunya virus which is transmitted to humans by Aedes mosquitoes. It poses a big threat and is likely to emerge in Bangladesh as a major public health problem after its first outbreak in December 2008. Since then, a couple of outbreaks were reported in different parts of the country. The first one was in Pabna upazilla in the Rajshahi district where 32 cases were identified. The second outbreak was in the Shathiya upazilla of Pabna in 2009. The third outbreak of Chikungunya fever has been discovered in the Dhaka, Dohar & Nababganj upazila of Dhaka district as well as in the Shibganj of Chapainababganj district which affected 46 persons (Chowdhury et al. 2012) (Fig.6.2D). There was no specific antiviral agent or vaccine against chikungunya available till recent years. Treatment is supportive and may involve rest, a proper diet, movement and mild exercise. Combination with mild pain relief medication, such as naproxen, ibuprofen, acetaminophen or paracetamol, it may relieve the fever and aches (Chowdhury et al. 2012). Re-evaluation and closer monitoring are advised in chronic ailments. Ideally, diagnosis of chikungunya fever should be based on isolation of the virus, molecular methods, detection of the IgM antibody, and demonstration of a rising titer of the IgG antibody. In Bangladesh, only detection of IgM Ab is currently possible in Dhaka city (Chowdhury et al. 2012).

Japanese encephalitis

Japanese encephalitis (JE) is a disease caused by the mosquito-borne Japanese encephalitis virus. In Bangladesh, the magnitude of Japanese encephalitis is largely unknown because of the limited diagnostic capacity and a lack of systematic disease surveillance. The only confirmed JE outbreak was documented in Bangladesh in 1977 (Khan et al. 1981). After the outbreak, a couple of hospital based encephalitis surveillance studies were conducted from 2003 to 2005 and in October 2007. It has been found, from the first study, that 20 out of 492 cases with clinical encephalitis, from four different hospitals located in Dhaka, Rajshahi, Mymensing and Sylhet division, had laboratory evidence of a recent JEV infection. Two deaths were documented in this study (Hossain et al. 2010). The second study found that the estimated JE incidence was 2.7/100,000 population in the Rajshahi Medical College Hospital catchment areas in Rajshahi division, 1.4/100,000 incidence in the Khulna Medical College Hospital catchment area in Khulna division and 0.6/100,000 incidence in the Chittagong Medical College Hospital in Chittagong Division (Paul, 2011) (Fig.6.2E). The vectors of JE—*Culex tritaeniorhynchus* and *Cx. gelidus*—have been found to be resistant to DDT (Mittal et al. 2004). Since Bangladesh shares a similar ecology with neighbouring countries India and Nepal, where several large outbreaks of JE have been identified in recent years, Bangladesh should consider a pilot project to introduce a JE vaccine in high-incidence areas (World Health Organisation 2012).

Visceral leishmaniasis

Endemicity:

Visceral leishmaniasis (VL), one of the most neglected protozoan systemic tropical diseases, is transmitted by female sand flies (*Phlebotomus argentipes*) (Yamey G 2000). In Bangladesh, VL is one of the major public health problems due to its strong association with poverty and ecological risk factors. Approximately, 4000–9000 VL patients have been identified in facility-based surveillance every year, and 18% of the population (20 million people) are considered to be at risk of VL (Rahman et al. 2010). However, after taking substantial under-reporting factors (such as lack of or poor diagnostic devices and an inadequate public health surveillance system in many parts of the highly-endemic areas) into account, estimated VL incidence in Bangladesh has increased from 12,400 to 24,900 cases per year (Alvar et al. 2012).

In Bangladesh, VL cases were reported sporadically in the 1970s, and an outbreak occurred in the Pabna district in 1980. Sirajganj, Pabna, Mymensingh, Rajshahi and Tangail were the districts mostly affected by VL in the early 1980s. From 1994 through 2004, a total of 73,467 VL cases were identified in these districts (Habib 2012).

Recently nine of the 47 endemic districts (Mymensingh, Tangail, Jamalpur, Gazipur, Sirajganj, Pabna, Nator, Naogaon, and Nawabgonj) were found to be highly endemic for VL (Fig. 6.2F). In the last 10 years (1999–2008), Mymensingh alone originates 60% of the total VL reported cases throughout the country. However, Pabna has reported the highest annual number of VL cases in 1994 to 1996. After 1996, the incidences in Mymensingh overtook those occurring in Pabna, and have continued to rise since that time (Bern & Chowdhury 2006).

In the Mymensingh district the Fulbaria, Trishal, Baluka, Muktagacha and Gaforgaon upazila are the most endemic thanas in recent years, with 30 – 33 incidents per 10,000 people per year rising to 5-15 incidents per 10,000 people per year since 2000 (Bern & Chowdhury 2006). Upazilas are the subdistricts of Bangladesh.

The “Visceral Leishmaniasis Elimination Programme” started in 2005 and a political commitment of the three neighbouring countries (India-Bangladesh- Nepal) tried to eliminate VL by 2015, this effort has shown a down-turn with VL cases rising from 2004 to 2013 - 16 upazilas showed case rates above the elimination target in which they ranged from 1.06 to 18.25 per 10,000 people per year (Bern & Chowdhury 2006).

Post-kala-azar dermal leishmaniasis (PKDL, which is a chronic maculopapular or nodular rash that occurs months to years after apparently successful VL treatment, serves as a

durable infection reservoir. It has been reported that PKDL incidence increased from 1 case per 10,000 person-years in 2002–2004 to 21 cases per 10,000 person-years in 2007 in Fulbaria in the Mymensingh district(Yamey G 2002; Alvar et al. 2012; S. Islam et al. 2013). These PKDL cases are important markers of transmission dynamics as they are supposedly highly infectious.VL Diagnosis in those cases is done via Rapid Diagnostic Tests (RDT with rk39 dipstick) for the diagnosis of cases by field workers.

Seasonality:

Though there is no significant difference in VL occurrence throughout the year, the peak of transmission (rise in the number of cases) has been reported in Pre-monsoon (March, April and May) and Post-monsoon (October and November) (Mondal et al. 2010).

Major pathogens:

Visceral leishmaniasis is caused by the parasites *Leishmania infantum*, *L. donovani* and *L. chagasi* . In Bangladesh, the disease is caused by *L. donovani* and affects both adults and children(Hossain et al. 1993) .

Vectors & insecticide resistance:

Of the 500 known *Phlebotomine* species, only around 30 of them have been positively identified as vectors of the disease (World Health Organisation 2009). *Phlebotomine argentipes* is the primary VL vector in Bangladesh(Ahluwalia et al. 2003). This species has been shown to be resistant to DDT in areas of VL transmission in Bihar, India, however, it is still susceptible to residual spraying of DDT in Bangladesh. Among non-vector species, *S.barraudi* is most abundant, followed by *S.babubabu*, *S.indica*, *S.perturbans*, *S.africanamagna*, *S.himalyensis* and *S.malabar-ica*. *S.b.babu* was the dominant species found in all localities in Bangladesh in 1997-97(Chowdhury et al. 2014).

Drugs:

Historically, antimonials have been the primary line of treatment for VL. Amphotericin B deoxycholate is more often used as a secondary line of treatment for VL cases responding poorly to antimonials as it requires prolonged hospitalization and repeated biochemical monitoring(Bern & Chowdhury 2006). Most VL in Bangladesh is still responsive to a Sodium Stibogluconate (SSG) regimen prescribed as a first line treatment, though the VL patient from India showed unresponsiveness to SSG drug (Bern, C; Chowdhury 2006;Sarker et al. 2003). Miltefosine is the only oral agent available and it has been recently recommended by the National Guidelines of Bangladesh (Banjara et al. 2012).

Risk factors for VL:

A study showed that VL risk was significant in persons 3-45 years of age who live in a VL patient's household or within 50 meters of a VL patient. Bed-net users, especially if the use is in summer, and an increased cattle density were shown to be a group under less risk. Education, income, land ownership or housing material and conditions. As well as keeping goats or chickens inside the bedroom have no effect on VL occurrence (Bern & Chowdhury 2006).

Bangladesh is one of the most climate vulnerable countries in the world, and it is expected to become even more so as a result of climate change. Projections indicate that vector borne diseases, especially malaria, VL and dengue fever will expand from established endemic areas. Furthermore, the intensity and extent of vector borne disease transmission will increase significantly in coming decades. In addition to this, quick changes in population dynamics, in terms of rapid urbanization, migration and growing urban slums, increase the chances of sudden outbreak of urban diseases like dengue. It is really difficult to predict the actual spread of these diseases in Bangladesh, especially in the case of neglected diseases such as JE and Lymphatic filariasis, due to the insufficient diagnostic facilities, amenities and a systematic study and data storage shortage. Recent documentation of substantial drug resistance, mutations in pathogens, insecticide resistance in the vector populations and significant outbreaks of neglected diseases originating in its main border sharing countries i.e, India and Myanmar, are putting extreme pressure on Bangladesh to look into the current situation of vector borne diseases in order to avoid any kind of abrupt outbreaks. Since all vector borne diseases reach their peak in the Monsoon, Integrated vector management (IVM) could be a rational approach for vector borne disease control.

Chapter 7

General Discussion

Anopheles coluzzii is a new member of *A. gambiae* complex. Previously, it was known as *A. gambiae* molecular form 'M'. There is no distinct morphological difference available in these two species. However, these two species exhibit biological differences i.e. *A. coluzzii* prefers long-standing man-made breeding sites whereas *A. gambiae* –S form is associated with ephemeral and rain-dependent breeding sites (della Torre et al. 2005; Kamdem et al. 2012; Lehmann & Diabate 2008). SNPs analysis (400,000) across the genomes of paired population samples of M and S form suggested that the two taxa are evolving collectively on independent evolutionary trajectories (Reidenbach et al. 2012). Moreover, pre-mating barriers present between M and S (Diabate et al. 2009; Pennetier et al. 2010). Based on these molecular and biological evidences *A. gambiae*-M form is assigned formal name *A. coluzzii* (COETZEE et al. 2013).

Stearoyl-CoA desaturase (SCD) - an iron-containing, microsomal enzyme, conserved in all eukaryotes – converts saturated fatty acids (SFAs) to mono unsaturated fatty acids (MUFAs). SCD is considered to be a pivotal enzyme in the body, since the ratio of stearic acid and oleic acid plays an important role in the maintenance of cell membrane fluidity and cell-cell interactions (Ntambi & Miyazaki, 2004). A literature review will show that, this thesis is the first report characterizing the *SCD1* gene phenotypically and genotypically in an insect model system.

Deregulation of the lipid metabolism, due to inhibition of key enzymes, especially SCD, acetyl co A carboxylase (ACC), or fatty acid synthase (FAS), which is involved in fatty acid and cholesterol biosynthesis, could lead to the development of various metabolic syndromes and physiological abnormalities, in mammals and insects (Ntambi & Miyazaki, 2004; Alabaster et al., 2011). The experiments, hereby undertaken, extend these observations, by finding that, the $\Delta 9$ -desaturase activity, and synthesis of the precursors (MUFA) of major structural components of biological membranes from SFA, appears to be essential for the survival, reproduction and co-ordinated blood meal digestion in adult, female *A. coluzzii*.

The findings of this thesis show that, *SCD1* inhibition in *A. coluzzii*, by both *dsSCD1* injection, and via a small biomolecule like sterculic acid (SA), has global effects on both genome and metabolism. Changes in gene expression and metabolite levels, owing to *SCD1* inhibition, were correlated with striking phenotypes in this model organism. *SCD1* inhibition changed the lipid distribution, both in *SCD1* KD and SA treated blood-fed mosquitoes. Blood-fed mosquito mortality was markedly higher compared to that of control mosquitoes, both in *SCD1* KD and SA treated mosquitoes (Chapter 3, Chapter 5). This overloading with fatty acids, especially SFAs, may induce a cellular toxic response, known as “lipotoxicity”, which develops a variety of diseases in the human body. Therefore, it has been hypothesized that, the perturbation of this process, following *SCD1* KD, leads to an accumulation of toxic SFAs, resulting in mosquito mortality, both in blood-fed and sugar-fed individuals. In this project, no experiment was performed to rescue this phenotype (mortality), by supplementing MUFA, in order to support the hypothesis that *SCD1* KD is entirely responsible for mosquito death and other phenotypes. Nevertheless, there are several studies which demonstrate that oleic acid supplementation can rescue *SCD1* inhibition effects in cells (Roongta et al. 2011; Nguyen et al. 2014).

The dramatic phenotype of blood meal-leakage into the mosquito body cavity, and a thicker lateral cell membrane (LCM), may suggest that, the integrity of the midgut membrane is compromised, as a result of *SCD1* silencing, and is the cause of accelerated mortality in blood-fed mosquitoes (Chapter 3). However, no blood-leakage was observed in SA treated mosquitoes. Additionally, no Electron microscopy was performed to analyse the midgut membrane of SA treated mosquitoes. Therefore, it is difficult to infer that, the mechanical stress, on the stiff cell membrane of the midgut, was also an influential contributor to mortality in SA treated mosquitoes.

In blood feeding mosquitoes, oocyte development involves the rapid accumulation of a large amount of fat, especially triacylglyceride following a blood meal; an oocyte contains 30-40% lipids. Dietary TAGs convert into monoacyl-glycerols and free fatty acids in the midgut lumen in the presence of lipases that produced by midgut cells. These free fatty acids enter into midgut cells and convert into diacylglycerol (DAG), triacylglycerol (TAG), and phospholipids in most of the insects(Canavoso et al. 2001). Fatty acids are promptly taken up by the fat body and are readily incorporated, chiefly into TAG and, in small amounts, into other glycerides and phospholipids. This loading and unloading of lipids into and from fat body is performed by a shuttle mechanism which involves LP and lipid transfer molecules. Lipophorin carries DAG to the ovarian tissues where these DAG molecules convert to TAG(Kawooya et al. 1986).

Since inhibition of SCD1 hinders the biosynthesis of TAG and cholesterol, which are the chief components of lipid droplets, and lipid droplets are involved in oocyte development, it is highly likely that, loss of SCD1 compromises the development of ovaries and oocytes, due to an insufficient amount of lipid droplets, in *A. coluzzii*. This conclusion rests on several lines of evidence, which correlate well with each other, namely an undeveloped ovaries scarcity of lipid droplets in the midgut and epithelial cells, and an undigested blood meal (Chapter 3). Moreover, mosquitoes fed on a mouse, treated with SA, laid significantly less eggs than the control mosquitoes, though the viability of these eggs was not checked in this experiment (Chapter 5). Thus, it would be interesting to study the development of eggs laid by SA treated mosquitoes, either through the use of a membrane feeder, or by following the same procedure mentioned in this thesis, in order to estimate the range of the effects of SCD1 inhibition in mosquitoes.

Gene expression profiling, of *SCD1* KD mosquitoes, exhibited a significant number of genes (601) which were differentially expressed, before and after blood meal ingestion. Blood meals trigger a complex program of gene expression within a few hours of consumption, which includes the activation and/or upregulation of several genes, required for the induction of physiological, morphological and hormonal changes in mosquitoes (Sodja et al. 2007). Consistent with this idea, at 6hr PBM, the number of regulated genes (141 genes) almost doubled in comparison to those exhibited at 0hr BBM; this may suggest that, the blood meal aggravated the consequences of *SCD1* KD in the mosquitoes' systems. Gene upregulation prevailed over gene downregulation in *SCD1* KD mosquitoes, especially in the immune response, signalling and cytoskeleton categories. Conversely, gene downregulation dominated over gene upregulation, at each time point, in *SCD1* KD mosquitoes in the metabolism, replication and detoxification categories (Chapter 4). Pathway analysis, on the basis of gene expression and metabolite accumulation, showed that *SCD1* KD lead to over-activation of β -oxidation and the TCA cycle as well as compromising the urea cycle, glycolysis, and amino acid synthesis. Whatever the underlying mechanism may be, alteration of the metabolic pathways, after SCD1 inhibition, lead to the excess accumulation of substrates of TCA cycle. The accumulation of various substrates may lead to the development of metabolic syndromes in mosquitoes, which cause mosquito death.

Metabolite profiles, however, highlight the overall similarity between *SCD1* KD mosquitoes and SA treated mosquitoes; there were only a few differences, such as those exhibited by glucose, and its derivatives, and by some amino acids (Chapter 4). This discordance, between the metabolites of *SCD1* KD mosquitoes and SA treated mosquitoes, may point to a difference in the method of action of *dsSCD1* and SA in the mosquito system. The findings of this thesis are insufficient in filling in these gaps in the knowledge, as there is no gene

expression data available, for SA treated mosquitoes. Therefore, it would be interesting to study global gene expressions, in SA treated mosquitoes, before and after blood feeding, in order to understand the effects of SA, on different metabolic and signalling pathways. Additionally, gene expression data is also required, to evaluate the off-target effects of this biomolecule on an organism, before proceeding with drug development.

It is notable that, disagreement between the transcription level of a gene (ODC) and the metabolite levels of its substrates was observed, in *SCD1* KD mosquitoes. This kind of disagreement can be resolved by in vivo measurement of the protein expression of this particular gene. Due to time constraints, this experiment was not performed (Chapter 4).

Upregulation of several inflammation marker genes, such as CEC1, CD80, FREPs and MD-2, in *SCD1* KD mosquitoes, indicates an induction of inflammation, triggered by altered lipid profiles. In this thesis, only CEC1 expression by qRT PCR was checked, in order to see if the over-activation of immunity genes, in *SCD1* KD mosquitoes, was exclusively dependent on *SCD1* KD and independent of midgut microbiota (Chapter 4). Nevertheless, it is important to check a number of immune gene expressions, in order to validate the hypothesis, that SFA accumulation, owing to *SCD1* KD, triggers an inflammatory signal in the mosquito system.

In brief, SA -a small bio molecule - has produced phenotypes such as mortality and compromised fecundity in adult *A. coluzzii*, which matches the phenotypes observed in *SCD1* KD mosquitoes. Apart from a few inconsistencies in metabolism, such as those seen in the glucose metabolism, the global metabolite profiling shows that SA treatment and *SCD1* KD, by RNAi, affect the same pathways, such as β -oxidation, the TCA cycle and TCA cycle-linked amino acid synthesis pathways. Moreover, SA treatment produced mortality in both adults and larvae of mosquitoes from different genera. This suggests that, the *SCD1* gene could be a potential novel target in mosquitoes, for the development of malarial interventions. It has been demonstrated that, SA or its derivatives have an effect on the viability of the asexual blood stages of *Plasmodium falciparum* (Gratraud et al., 2009) or reproduction of the hepatitis C virus, which a member of the family Flaviviridae, to which the dengue virus also belong (L. N. Nguyen et al. 2014).

Considering all this evidence, it can be hypothesized that, *SCD1* is not only a good target for insecticide development, but also for the development of antimalarial or anti-dengue drugs. These drugs may benefit human hosts by killing disease agents, as well as controlling the vector population, by passively affecting the fecundity of the mosquitoes, which come to feed on the affected humans.

It has been demonstrated that, SA, or its derivatives, have an effect on the viability of the asexual blood stage of *Plasmodium falciparum* (Gratraud et al. 2009) which is the causative agent of malaria, and on the reproduction of the hepatitis C virus, which a member of the family Flaviviridae, to which dengue virus also belongs (L. N. Nguyen et al. 2014). The findings of this thesis are consistent with these studies and suggest that, SA has a lethal effect, not only the adults of *A. coluzzii*, but also in *Ae. aegypti* adults and larvae. Adult mosquitoes, from two different genera, showed significant mortality, after taking a blood meal supplemented with SA. This primary, fascinating findings suggest that, SCD1 can be a potential target for malaria intervention. However, the effective concentration of SA, used in this experiment, was very high (1mM). This needs to be reduced to a reasonable concentration, in order to develop efficient insecticides or an antimalarial drug. There is a possibility that, the buffer used to dissolve SA was not efficient, therefore, a higher concentration of SA was required to achieve IC_{50} . This issue needs to be addressed in the future, in order to develop commercial insecticides, targeting SCD1 in mosquitoes. Considering all these strands of evidence, it can be hypothesized that, SCD1 might be a good target for insecticide development.

Chapter 6 of the thesis, a review of the current situation of vectorborne diseases in Bangladesh which are basically non-existent in the relevant literature, put forward a solid background of further research on vectorborne diseases in Bangladesh¹. Climate change - a complex system of environmental changes, occurring around the world, with unknown future risks to human and natural ecosystems - has a significant effect on vector-borne diseases. In light of this issue, developing countries are at a high risk of public health disasters, due to a combination of rapid population growth, urbanization, economic instability and chemical pollution. Bangladesh is one of the most climate-vulnerable countries in the world, and it is expected to become even more so, as a result of climate change. The mortality and morbidity rates are highest for malaria among all other vector-borne diseases, followed by VL. However, recent changes in population dynamics and leaving standards in terms of urbanization, migration and growing urban slums have drastically increased the possibility for outbreaks of urban diseases such as DF. This together with recent reports of drug resistant pathogens, insecticide resistant vector populations and outbreaks of neglected diseases in neighbouring countries including India and Myanmar cause an alarming situation and stress the need for a national strategy to deal with potential significant outbreaks in the future. Furthermore, the intensity and extent of vector-borne disease transmission will increase significantly, in coming decades.

¹ In the chapter 6 of this thesis, the author reviewed the current situation of vectorborne diseases in Bangladesh as a part of the requirements from her scholarship body (Commonwealth Scholarship commission)

Future Direction

The findings of this thesis have shed light on the functions of SCD1 in *A.coluzzii*. This thesis investigated the phenotypic, molecular and biochemical consequences of SCD1 inhibition in *A. coluzzii* - a model organism. These studies report the pattern of changes, in the gene expression and metabolite profile both in *SCD1* KD and SA treated mosquitoes, which lead to striking phenotypes, in response to an altered desaturase index (SFA/MUFA ratio) in mosquitoes. These findings contribute to the present knowledge of the lipid metabolism in insects, but there are still questions to be answered. The mechanisms behind the independent associations, between SCD1 activities and genetic and metabolic regulation, should be investigated in future studies. Particularly, the regulation of the insulin pathway, in response to *SCD1* KD in insects, could be an interesting target for further investigation, since IIP pathways modulate the expression of the vitellogenin gene in mosquitoes. The primary findings of this thesis point to an inflammation in *SCD1* KD mosquitoes. It would be thought-provoking to study the mechanism of inflammation induction, in the insect system, using *A. coluzzii* as a model organism, in order to better understand its biology.

The target of rapamycin (TOR, or mTOR for mammalian TOR) is a candidate pathway for involvement in the regulation of the lipid metabolism, which is conserved from yeast to mammals. It controls the gene expression and protein biosynthesis for cell growth, proliferation, motility and survival via transcriptional and translational regulatory pathways (Rohde 2001). The TOR signalling pathway plays a crucial role in *Ae. aegypti* reproduction, as it increases vitellogenin expression in the fat body, in response to amino acid abundance and the activation of egg development, after a blood meal (Hansen, 2005). The relationship between this TOR-signalling pathway and SCD1 has been established, in mammalian breast cancer cells (Luyimbazi 2001). In their study, Luyimbazi showed that rapamycin (an mTOR inhibitor) and its orthologs decrease SCD1 transcriptome expression, by inhibiting SCD1 translation initiation factor-binding protein (4EBP1) and Sterol regulatory element binding protein1 (SREBP1), which may occur through the mTOR/eIF4E-binding protein 1 axis. Thus, it would be interesting to study the interaction between the SCD1 and TOR signalling pathways in the future, with the aim of developing new malarial interventions.

SA has produced phenotypes such as mortality and compromised fecundity in adult *A. coluzzii*, which matches the phenotypes observed in *SCD1* KD mosquitoes. However, the effective concentration of SA, used in this experiment, was very high (1mM). Apart from few inconsistencies in metabolism, such as those seen in the glucose metabolism, the global metabolite profiling shows that SA treatment and *SCD1* KD by RNAi, affect the same pathways, such as β -oxidation, the TCA cycle- linked amino acid synthesis pathways. Thus

it would be interesting to investigate whether SCD1 gene can be used as a potential novel molecular target in mosquito for the development of malaria intervention or not. To achieve this goal 'gene to lead' and 'lead to product' phases can be done (Meyer et al. 2012). In 'gene to lead phase' the expression of *SCD1* gene in eggs, larvae, pupae, adult male and female mosquitoes can be conducted as a part of target validation. The quantitative correlation between the pharmacological, ecological and toxicological activities to the structure of SA can be done in future in order to predict the "desire efficacy" of structurally related compounds. Pharmacological characterization is also required for target validation. If all these experiments suggest that *SCD* is a potential target for insecticide development, in vivo high throughput screening can be performed to screen large amounts of chemical compounds against this SCD1. Before field trial, ultra-high-throughput in vivo screening (UHTVS) from the natural substances will be needed to detect appropriate insecticidal efficacy.

Chapter 7

Summary

Ingestion of blood by female mosquitoes is a pre-requisite for both egg production and transmission of blood-borne pathogens. Ingested blood meal proteins including haemoglobin are converted to lipids and stored in the fat body for where they are transported to the developing eggs. This thesis aimed to functionally and phenotypically characterise the stearoyl- CoA desaturase (SCD1), a critical enzyme in the lipid metabolism pathway, in the mosquito *A. coluzzii*, one of the two main vectors of malaria in sub-Saharan Africa. In addition to generating storage lipids, this enzyme is essential for the maintenance of cell membrane fluidity and other housekeeping functions. Silencing of the gene encoding SCD1 and inhibition of the SCD1 function using a known drug causes a multitude of physiological and metabolic syndromes including blood perfusion of the haemocoel, inhibition of egg development, cell membrane integrity and acute inflammation, which together lead to premature mosquito death soon after human blood ingestion. Profiling of the transcriptional and metabolite changes following SCD1 loss-of-function in conjunction with biochemical

pathway analysis revealed a biochemical framework in which SCD1 functions following a blood meal. These findings could provide important leads for the development of novel vector control interventions.

Chapter 8

Bibliography

- Abe, H. et al., 1985. Role of histidine-related compounds to intracellular buffering in fish skeletal muscle. *The American journal of physiology*, 249(4 Pt 2), pp.R449–54. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/4051030>.
- Aboagye-Antwi, F. et al., 2015. Experimental Swap of *Anopheles gambiae*'s Assortative Mating Preferences Demonstrates Key Role of X-Chromosome Divergence Island in Incipient Sympatric Speciation B. A. Payseur, ed. *PLOS Genetics*, 11(4), p.e1005141. Available at: <http://dx.plos.org/10.1371/journal.pgen.1005141>.
- Ahasan, H. a M.N. et al., 2008. Review Articles Treatment of Kala-Azar : Past , Present & Future. *J MEDICINE*, 9, pp.90–95.
- Ahluwalia, I.B. et al., 2003. Visceral leishmaniasis: Consequences of a neglected disease in a Bangladeshi community. *American Journal of Tropical Medicine and Hygiene*, 69(6), pp.624–628.
- Ahmed, S. et al., 2013. Malaria Hotspots Drive Hypoendemic Transmission in the Chittagong Hill Districts of Bangladesh. *PLoS ONE*, 8(8).
- Ahmed, U., 1987. Checklist of the mosquitos of Bangladesh. *Mosquito Systematics*, 19(3), pp.187–200.
- Alabaster, A. et al., 2011. Deficiencies in acetyl-CoA carboxylase and fatty acid synthase 1 differentially affect eggshell formation and blood meal digestion in *Aedes aegypti*. *Insect Biochemistry and Molecular Biology*, 41(12), pp.946–955. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0965174811001755>.
- Alam, A.S. et al., 2010. Clinical Profile of Dengue Fever in Children. *Bangladesh Journal of Child Health*, 33(2). Available at: <http://banglajol.info/index.php/BJCH/article/view/5678>.
- Alam, M. et al., 2012. Diversity of anopheline species and their Plasmodium infection status in rural Bandarban, Bangladesh. *Parasites & Vectors*, 5(1), p.150. Available at: ???
- Ali, M. et al., 2003. Use of a geographic information system for defining spatial risk for dengue transmission in Bangladesh: Role for *Aedes albopictus* in an urban outbreak. *American Journal of Tropical Medicine and Hygiene*, 69(6), pp.634–640.
- Alpey, L. et al., 2010. Sterile-Insect Methods for Control of Mosquito-Borne Diseases: An Analysis. *Vector-Borne and Zoonotic Diseases*, 10(3), pp.295–311. Available at: <http://www.liebertonline.com/doi/abs/10.1089/vbz.2009.0014>.
- Alvar, J. et al., 2012. Leishmaniasis worldwide and global estimates of its incidence. *PLoS ONE*, 7(5).
- Arrese, E.L. & Soulages, J.L., 2010. Insect fat body: energy, metabolism, and regulation. *Annual Review of Entomology*, 55(87), pp.207–225.
- Arrese, E.L. & Soulages, J.L., 2010. Insect Fat Body: Energy, Metabolism, and Regulation. *Annual Review of Entomology*, 55(1), pp.207–225. Available at:

<http://www.annualreviews.org/doi/abs/10.1146/annurev-ento-112408-085356>.

- Atella, G.C. et al., 2006. Anopheles gambiae lipophorin: Characterization and role in lipid transport to developing oocyte. *Insect Biochemistry and Molecular Biology*, 36(5), pp.375–386.
- Attardo, G.M. et al., 2006. Identification of two cationic amino acid transporters required for nutritional signaling during mosquito reproduction. *The Journal of experimental biology*, 209(Pt 16), pp.3071–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16888056>.
- Attie, A.D. et al., 2002. Relationship between stearyl-CoA desaturase activity and plasma triglycerides in human and mouse hypertriglyceridemia. *Journal of lipid research*, 43(11), pp.1899–907. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12401889>.
- Austic, R.E., Hill, F.W. & Wilson, B.W., 1971. Role of Yolk in Chick Embryo Mortality Induced by the Feeding of Sterculia Foetida Oil. *Poultry Science*, 50(5), pp.1320–1326. Available at: <http://ps.oxfordjournals.org/cgi/doi/10.3382/ps.0501320>.
- Ayala, F.J. & Coluzzi, M., 2005. Chromosome speciation: Humans, Drosophila, and mosquitoes. *Proceedings of the National Academy of Sciences*, 102(Supplement 1), pp.6535–6542. Available at: <http://www.pnas.org/cgi/doi/10.1073/pnas.0501847102>.
- Baenke, F. et al., 2013. Hooked on fat: the role of lipid synthesis in cancer metabolism and tumour development. *Disease Models & Mechanisms*, 6(6), pp.1353–1363. Available at: <http://dmm.biologists.org/cgi/doi/10.1242/dmm.011338>.
- Bai, Y. et al., 2015. X-ray structure of a mammalian stearyl-CoA desaturase. *Nature*, 524(7564), pp.252–6. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/26098370>.
- Baldini, F. et al., 2014. Evidence of natural Wolbachia infections in field populations of Anopheles gambiae. *Nature communications*, 5, p.3985. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24905191>.
- Bangladesh Bureau of Statistics, 2013. *STATISTICAL POCKETBOOK OF BANGLADESH-2013*,
- Banjara, M.R. et al., 2012. Visceral Leishmaniasis Clinical Management in Endemic Districts of India, Nepal, and Bangladesh. *Journal of Tropical Medicine*, 2012, pp.1–8. Available at: <http://www.hindawi.com/journals/jtm/2012/126093/>.
- Ben-David, U. et al., 2013. Selective elimination of human pluripotent stem cells by an oleate synthesis inhibitor discovered in a high-throughput screen. *Cell stem cell*, 12(2), pp.167–79. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23318055>.
- Bern, C; Chowdhury, R., 2006. The epidemiology of visceral leishmaniasis in Bangladesh: prospects for improved control. *Indian J Med Res*, 123(3), pp.275–88.
- Bern, C. & Chowdhury, R., 2006. The epidemiology of visceral leishmaniasis in Bangladesh: Prospects for improved control. *Indian Journal of Medical Research*, 123(3), pp.275–288.
- Bickel, P.E., Tansey, J.T. & Welte, M.A., 2009. PAT proteins, an ancient family of lipid droplet proteins that regulate cellular lipid stores. *Biochimica et biophysica acta*, 1791(6), pp.419–40. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19375517>.
- BINDER, R.G. & CHAN, B.G., 1982. EFFECTS OF CYCLOPROPANOIC AND CYCLOPROPENOIC FATTY ACIDS ON GROWTH OF PINK BOLLWORM, BOLLWORM AND TOBACCO BUDWORM. *Entomologia Experimentalis et Applicata*,

- 31(2-3), pp.291–295. Available at: <http://doi.wiley.com/10.1111/j.1570-7458.1982.tb03147.x>.
- Blacklock, B.J. & Ryan, R.O., 1994. Hemolymph lipid transport. *Insect Biochemistry and Molecular Biology*, 24(9), pp.855–873. Available at: <http://linkinghub.elsevier.com/retrieve/pii/0965174894900159>.
- Blandin, S. et al., 2002. Reverse genetics in the mosquito *Anopheles gambiae*: targeted disruption of the Defensin gene. *EMBO reports*, 3(9), pp.852–6. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12189180>.
- Blanford, S. et al., 2005. Fungal pathogen reduces potential for malaria transmission. *Science (New York, N.Y.)*, 308(5728), pp.1638–41. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15947189>.
- Boura, E. & Nencka, R., 2015. Phosphatidylinositol 4-kinases: Function, structure, and inhibition. *Experimental Cell Research*, 337(2), pp.136–145. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0014482715002323>.
- Bowen, M.F., 1992. Patterns of sugar feeding in diapausing and nondiapausing *Culex pipiens* (Diptera: Culicidae) females. *Journal of medical entomology*, 29(5), pp.843–9. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/1404264>.
- Briand, L. et al., 2001. Ligand binding and physico-chemical properties of ASP2, a recombinant odorant-binding protein from honeybee (*Apis mellifera* L.). *European journal of biochemistry / FEBS*, 268(3), pp.752–60. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11168415>.
- Brown, J.M. & Rudel, L.L., 2010. Stearoyl-coenzyme A desaturase 1 inhibition and the metabolic syndrome: considerations for future drug discovery. *Current Opinion in Lipidology*, 21(3), pp.192–197. Available at: <http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00041433-201006000-00006>.
- Bryant, C.E. et al., 2010. The molecular basis of the host response to lipopolysaccharide. *Nature reviews. Microbiology*, 8(1), pp.8–14. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19946286>.
- Canavoso, L.E. et al., 2001. Fat metabolism in insects. *Annual Review of Nutrition*, 21(1), pp.23–46. Available at: <http://www.annualreviews.org/doi/abs/10.1146/annurev.nutr.21.1.23>.
- Carpenter, V.K. et al., 2012. SLC7 amino acid transporters of the yellow fever mosquito *Aedes aegypti* and their role in fat body TOR signaling and reproduction. *Journal of insect physiology*, 58(4), pp.513–22. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22266018>.
- Chahar, H.S. et al., 2009. Co-infections with chikungunya virus and dengue virus in Delhi, India. *Emerging infectious diseases*, 15(7), pp.1077–80. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19624923>.
- Chajès, V., Joulin, V. & Clavel-Chapelon, F., 2011. The fatty acid desaturation index of blood lipids, as a biomarker of hepatic stearoyl-CoA desaturase expression, is a predictive factor of breast cancer risk. *Current opinion in lipidology*, 22(1), pp.6–10. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20935562>.
- Chen, C. et al., 2008. Metabolomics Reveals that Hepatic Stearoyl-CoA Desaturase 1

- Downregulation Exacerbates Inflammation and Acute Colitis. *Cell Metabolism*, 7(2), pp.135–147. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S1550413107003762>.
- Chiba, S., 1997. Molecular mechanism in alpha-glucosidase and glucoamylase. *Bioscience, biotechnology, and biochemistry*, 61(8), pp.1233–9. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/9301101>.
- Choi, Y.-J. et al., 2012. Tissue-enriched expression profiles in *Aedes aegypti* identify hemocyte-specific transcriptome responses to infection. *Insect Biochemistry and Molecular Biology*, 42(10), pp.729–738. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0965174812000872>.
- Chowdhury, R. et al., 2014. How dengue vector *Aedes albopictus* (Diptera: Culicidae) survive during the dry season in Dhaka City, Bangladesh? *Journal of vector borne diseases*, 51(3), pp.179–87. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25253210>.
- Christophides, G.K., Vlachou, D. & Kafatos, F.C., 2004. Comparative and functional genomics of the innate immune system in the malaria vector *Anopheles gambiae*. *Immunological reviews*, 198, pp.127–48. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15199960>.
- COETZEE, M. et al., 2013. *Anopheles coluzzii* and *Anopheles amharicus*, new members of the *Anopheles gambiae* complex. *Zootaxa*, 3619(3). Available at: <http://biotaxa.org/Zootaxa/article/view/zootaxa.3619.3.2>.
- Cohen, E., 2001. Chitin synthesis and inhibition: a revisit. *Pest Management Science*, 57(10), pp.946–950. Available at: <http://doi.wiley.com/10.1002/ps.363>.
- Colpitts, T.M. et al., 2012. West Nile Virus: biology, transmission, and human infection. *Clinical microbiology reviews*, 25(4), pp.635–48. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23034323>.
- Coluzzi, M., Petrarca, V. & di Deco, M.A., 1985. Chromosomal inversion intergradation and incipient speciation in *Anopheles gambiae*. *Bolletino di zoologia*, 52(1-2), pp.45–63. Available at: <http://www.tandfonline.com/doi/abs/10.1080/11250008509440343>.
- Conejero-Goldberg, C. et al., 2005. Infectious pathogen detection arrays: viral detection in cell lines and postmortem brain tissue. *BioTechniques*, 39(5), pp.741–51. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16312221>.
- Cook, H. & McMaster, C., 2002. Fatty acid desaturation and chain elongation in eukaryotes. *Biochemistry of Lipids, Lipoproteins and Membranes*, pp.181–204. Available at: <http://www.sciencedirect.com/science/article/pii/S0167730608605128>.
- Crawford, J.E. et al., 2015. Reticulate Speciation and Barriers to Introgression in the *Anopheles gambiae* Species Complex. *Genome Biology and Evolution*, 7(11), pp.3116–3131. Available at: <http://gbe.oxfordjournals.org/lookup/doi/10.1093/gbe/evv203>.
- Cunha-Rodrigues, M. et al., 2008. Genistein-Supplemented Diet Decreases Malaria Liver Infection in Mice and Constitutes a Potential Prophylactic Strategy D. Fox, ed. *PLoS ONE*, 3(7), p.e2732. Available at: <http://dx.plos.org/10.1371/journal.pone.0002732>.
- Deutschman, A.J. et al., 1961. Elimination of Pink White Discoloration in Stored Eggs from Hens Fed Treated Cottonseed Meal or Sterculic Acid. *Poultry Science*, 40(5), pp.1305–1310. Available at: <http://ps.oxfordjournals.org/cgi/doi/10.3382/ps.0401305>.

- Diallo, M., Thonnon, J. & Fontenille, D., 2000. Vertical transmission of the yellow fever virus by *Aedes aegypti* (Diptera, Culicidae): dynamics of infection in F1 adult progeny of orally infected females. *The American journal of tropical medicine and hygiene*, 62(1), pp.151–6. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10761742>.
- Ding, H. et al., 2015. Transformation of multi-component ginkgolide into ginkgolide B by *Coprinus comatus*. *BMC Biotechnology*, 15(1), p.17. Available at: <http://www.biomedcentral.com/1472-6750/15/17>.
- Doble, B.W. & Woodgett, J.R., 2003. GSK-3: tricks of the trade for a multi-tasking kinase. *Journal of cell science*, 116(Pt 7), pp.1175–86. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12615961>.
- Dong, Y. et al., 2006a. Anopheles gambiae Immune Responses to Human and Rodent Plasmodium Parasite Species. *PLoS Pathogens*, 2(6), p.e52. Available at: <http://dx.plos.org/10.1371/journal.ppat.0020052>.
- Dong, Y. et al., 2006b. Anopheles gambiae immune responses to human and rodent Plasmodium parasite species. *PLoS pathogens*, 2(6), p.e52. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16789837>.
- Dong, Y. & Dimopoulos, G., 2009. Anopheles Fibrinogen-related Proteins Provide Expanded Pattern Recognition Capacity against Bacteria and Malaria Parasites. *Journal of Biological Chemistry*, 284(15), pp.9835–9844. Available at: <http://www.jbc.org/cgi/doi/10.1074/jbc.M807084200>.
- Eisele, T.A. et al., 1979. Incorporation of label from [9, 10-methylene-14C] sterculic acid in rainbow trout, *Salmo gairdneri*. *Lipids*, 14(6), pp.523–528. Available at: <http://link.springer.com/10.1007/BF02533525>.
- Elahi, R. et al., 2013. Performance of a HRP-2/pLDH based rapid diagnostic test at the Bangladesh-India-Myanmar border areas for diagnosis of clinical malaria. *Malaria journal*, 12(1), p.378. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24172045>.
- Faruque, L.I. et al., 2012. Hospital-Based Prevalence of Malaria and Dengue in Febrile Patients in Bangladesh. *American Journal of Tropical Medicine and Hygiene*, 86(1), pp.58–64. Available at: <http://www.ajtmh.org/cgi/doi/10.4269/ajtmh.2012.11-0190>.
- Fontenille, D. et al., 1998. New vectors of Rift Valley fever in West Africa. *Emerging infectious diseases*, 4(2), pp.289–93. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/9621201>.
- Foster, W. a, 1995. Mosquito sugar feeding and reproductive energetics. *Annual review of entomology*, 40, pp.443–474.
- Fuchs, S. et al., 2014. Phenylalanine metabolism regulates reproduction and parasite melanization in the malaria mosquito. *PloS one*, 9(1), p.e84865. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24409310>.
- Fuehrer, H.P. et al., 2014. High prevalence and genetic diversity of Plasmodium malariae and no evidence of Plasmodium knowlesi in Bangladesh. *Parasitology Research*, 113(4), pp.1537–1543.
- Fuehrer, H.P. et al., 2010. Indigenous Plasmodium ovale malaria in Bangladesh. *American Journal of Tropical Medicine and Hygiene*, 83(1), pp.75–78.
- Fuehrer, H.P. et al., 2011. Novel nested direct PCR technique for malaria diagnosis using filter paper samples. *Journal of Clinical Microbiology*, 49(4), pp.1628–1630.

- Fuehrer, H.P. et al., 2012. Plasmodium ovale in Bangladesh: Genetic diversity and the first known evidence of the sympatric distribution of Plasmodium ovale curtisi and Plasmodium ovale wallikeri in southern Asia. *International Journal for Parasitology*, 42(7), pp.693–699. Available at: <http://dx.doi.org/10.1016/j.ijpara.2012.04.015>.
- Gaio, A. de O. et al., 2011. Contribution of midgut bacteria to blood digestion and egg production in aedes aegypti (diptera: culicidae) (L.). *Parasites & vectors*, 4, p.105. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21672186>.
- Gangl, E. et al., 2002. Structural elucidation of metabolites of ritonavir and indinavir by liquid chromatography–mass spectrometry. *Journal of Chromatography A*, 974(1-2), pp.91–101. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0021967302012438>.
- Gare, D.C., Piertney, S.B. & Billingsley, P.F., 2003. Anopheles gambiae collagen IV genes: cloning, phylogeny and midgut expression associated with blood feeding and Plasmodium infection. *International journal for parasitology*, 33(7), pp.681–90. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12814648>.
- Getz, G., Levine, E. & Domany, E., 2000. Coupled two-way clustering analysis of gene microarray data. *Proceedings of the National Academy of Sciences of the United States of America*, 97(22), pp.12079–84. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11035779>.
- Ghosh, A., Edwards, M.J. & Jacobs-Lorena, M., 2000. The journey of the malaria parasite in the mosquito: hopes for the new century. *Parasitology today (Personal ed.)*, 16(5), pp.196–201. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10782078>.
- Gondim, K.C. et al., 1992. Role of phospholipids in the lipophorin particles of Rhodnius prolixus. *Archives of Insect Biochemistry and Physiology*, 20(4), pp.303–314. Available at: <http://doi.wiley.com/10.1002/arch.940200406>.
- Gratraud, P. et al., 2009. Oleic Acid Biosynthesis in Plasmodium falciparum: Characterization of the Stearoyl-CoA Desaturase and Investigation as a Potential Therapeutic Target M. J. Horsburgh, ed. *PLoS ONE*, 4(9), p.e6889. Available at: <http://dx.plos.org/10.1371/journal.pone.0006889>.
- Grimstad, P.R. & DeFoliart, G.R., 1974. Nectar Sources of Wisconsin Mosquitoes. *Journal of Medical Entomology*, 11(3), pp.331–341. Available at: <http://jme.oxfordjournals.org/cgi/doi/10.1093/jmedent/11.3.331>.
- Gu, X. et al., 2010. Bip overexpression, but not CHOP inhibition, attenuates fatty-acid-induced endoplasmic reticulum stress and apoptosis in HepG2 liver cells. *Life Sciences*, 87(23-26), pp.724–732. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0024320510004601>.
- Habtewold, T. et al., 2008. Transmission Blocking Immunity in the Malaria Non-Vector Mosquito Anopheles quadriannulatus Species A D. S. Schneider, ed. *PLoS Pathogens*, 4(5), p.e1000070. Available at: <http://dx.plos.org/10.1371/journal.ppat.1000070>.
- Hamby, K.A. et al., 2013. Integrating Circadian Activity and Gene Expression Profiles to Predict Chronotoxicity of Drosophila suzukii Response to Insecticides M. F. Feldlaufer, ed. *PLoS ONE*, 8(7), p.e68472. Available at: <http://dx.plos.org/10.1371/journal.pone.0068472>.
- Handel, E. Van, 1984. metabolism of nutrients in the adult mosquito. *Mosquito news*, 44(4), pp.573–584.

- Hänscheid, T., 2003. Current strategies to avoid misdiagnosis of malaria. *Clinical Microbiology and Infection*, 9(6), pp.497–504.
- Hansen, I.A. et al., 2005. Target of rapamycin-dependent activation of S6 kinase is a central step in the transduction of nutritional signals during egg development in a mosquito. *The Journal of biological chemistry*, 280(21), pp.20565–72. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15788394>.
- Hansen, I.A. et al., 2004. Target of rapamycin-mediated amino acid signaling in mosquito anautogeny. *Proceedings of the National Academy of Sciences of the United States of America*, 101(29), pp.10626–31. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15229322>.
- Haque, A. et al., 2011. Type I interferons suppress CD4 + T-cell-dependent parasite control during blood-stage Plasmodium infection. *European Journal of Immunology*, 41(9), pp.2688–2698.
- Haque, U., Ahmed, S.M., et al., 2009. Malaria prevalence in endemic districts of Bangladesh. *PLoS ONE*, 4(8), pp.1–9.
- Haque, U. et al., 2012. Modelling malaria treatment practices in Bangladesh using spatial statistics. *Malaria Journal*, 11(1), p.63. Available at: <http://www.malariajournal.com/content/11/1/63>.
- Haque, U., Huda, M., et al., 2009. Spatial malaria epidemiology in Bangladeshi highlands. *Malaria journal*, 8, p.185.
- Hay, N., 2004. Upstream and downstream of mTOR. *Genes & Development*, 18(16), pp.1926–1945. Available at: <http://www.genesdev.org/cgi/doi/10.1101/gad.1212704>.
- Heinemann, F.S. & Ozols, J., 2003. Stearoyl-CoA desaturase, a short-lived protein of endoplasmic reticulum with multiple control mechanisms. *Prostaglandins, leukotrienes, and essential fatty acids*, 68(2), pp.123–33. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12538076>.
- Hemingway, J., 2002. An Overview of Insecticide Resistance. *Science*, 298(5591), pp.96–97. Available at: <http://www.sciencemag.org/cgi/doi/10.1126/science.1078052>.
- Hernando, J. et al., 2002. Synthesis of sterculic acid. *Arkivoc*, 2002(v), pp.26–30.
- Hill, C.A. et al., 2005. Arthropod-borne diseases: vector control in the genomics era. *Nature reviews. Microbiology*, 3(3), pp.262–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15703759>.
- Van der Horst, D.J., 1990. Lipid transport function of lipoproteins in flying insects. *Biochimica et biophysica acta*, 1047(3), pp.195–211. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/2252909>.
- HossainMI, Khan, S.A., Ameen, M., 1993. Phlebotomine sandflies of Bangladesh: recent surveys. *Med. Vet. Entomol*, 7, pp.99–101.
- Hotez, P.J. et al., 2006. Incorporating a rapid-impact package for neglected tropical diseases with programs for HIV/AIDS, tuberculosis, and malaria. *PLoS medicine*, 3(5), p.e102. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16435908>.
- Howard, R.W. & Blomquist, G.J., 2005. ECOLOGICAL, BEHAVIORAL, AND BIOCHEMICAL ASPECTS OF INSECT HYDROCARBONS*. *Annual Review of Entomology*, 50(1), pp.371–393. Available at:

- <http://www.annualreviews.org/doi/abs/10.1146/annurev.ento.50.071803.130359>.
- Hua, G. et al., 2008. Anopheles gambiae Cadherin AgCad1 Binds the Cry4Ba Toxin of *Bacillus thuringiensis israelensis* and a Fragment of AgCad1 Synergizes Toxicity †. *Biochemistry*, 47(18), pp.5101–5110. Available at: <http://pubs.acs.org/doi/abs/10.1021/bi7023578>.
- Huang, F.-F. et al., 2008. The UDP-glucosyltransferase multigene family in *Bombyx mori*. *BMC Genomics*, 9(1), p.563. Available at: <http://www.biomedcentral.com/1471-2164/9/563>.
- Husson, A. et al., 2003. Argininosuccinate synthetase from the urea cycle to the citrulline-NO cycle. *European journal of biochemistry / FEBS*, 270(9), pp.1887–99. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12709047>.
- Hüttemann, M. et al., 2012. Regulation of mitochondrial respiration and apoptosis through cell signaling: cytochrome c oxidase and cytochrome c in ischemia/reperfusion injury and inflammation. *Biochimica et biophysica acta*, 1817(4), pp.598–609. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21771582>.
- Igal, R.A., 2011. Roles of StearoylCoA Desaturase-1 in the Regulation of Cancer Cell Growth, Survival and Tumorigenesis. *Cancers*, 3(4), pp.2462–2477. Available at: <http://www.mdpi.com/2072-6694/3/2/2462/>.
- Ingrid V. F. van den Broek, Sophia van der Wardt, Lenin Talukder, Swarupa Chakma, A.B. & Anderson, S.N. and T.C., 2004. Drug resistance in *Plasmodium falciparum* from the Chittagong Hill Tracts, Bangladesh. *Tropical Medicine and International Health*, 9(6), pp.680–687.
- Islam, N., Bonovas, S. & Nikolopoulos, G.K., 2013. An epidemiological overview of malaria in Bangladesh. *Travel Medicine and Infectious Disease*, 11(1), pp.29–36. Available at: <http://dx.doi.org/10.1016/j.tmaid.2013.01.004>.
- Islam, S. et al., 2013. Clinical and immunological aspects of post-kala-azar dermal leishmaniasis in Bangladesh. *American Journal of Tropical Medicine and Hygiene*, 89(2), pp.345–353.
- Isoe, J. et al., 2011. Defects in coatamer protein I (COPI) transport cause blood feeding-induced mortality in Yellow Fever mosquitoes. *Proceedings of the National Academy of Sciences*, 108(24), pp.E211–E217. Available at: <http://www.pnas.org/cgi/doi/10.1073/pnas.1102637108>.
- Jambulingam P, Mohapatra SS, Das LK, Das PK, R.P., 1989. Detection of *Plasmodium ovale* in Koraput district, Orissa state. *Indian J Med Res.*, 89, pp.115–6.
- Jansen, C.C. et al., 2008. Vector competence of Australian mosquito species for a North American strain of West Nile virus. *Vector borne and zoonotic diseases (Larchmont, N.Y.)*, 8(6), pp.805–11. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/18973445>.
- Juneja, P. et al., 2015. Exome and Transcriptome Sequencing of *Aedes aegypti* Identifies a Locus That Confers Resistance to *Brugia malayi* and Alters the Immune Response. *PLOS Pathogens*, 11(3), p.e1004765. Available at: <http://dx.plos.org/10.1371/journal.ppat.1004765>.
- Kamdem, C. et al., 2012. Anthropogenic Habitat Disturbance and Ecological Divergence between Incipient Species of the Malaria Mosquito *Anopheles gambiae* J. Pinto, ed. *PLoS ONE*, 7(6), p.e39453. Available at:

<http://dx.plos.org/10.1371/journal.pone.0039453>.

Karim, M.N. et al., 2012. Climatic factors influencing dengue cases in Dhaka city: A model for dengue prediction. *Indian Journal of Medical Research*, 136(1), pp.32–39.

Kawooya, J.K. et al., 1986. Physical and surface properties of insect apolipoprotein III. *The Journal of biological chemistry*, 261(29), pp.13588–91. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/3759983>.

Kerner, J. & Hoppel, C., 2000. Fatty acid import into mitochondria. *Biochimica et biophysica acta*, 1486(1), pp.1–17. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10856709>.

Kikawada, T. et al., 2007. Trehalose transporter 1, a facilitated and high-capacity trehalose transporter, allows exogenous trehalose uptake into cells. *Proceedings of the National Academy of Sciences*, 104(28), pp.11585–11590. Available at: <http://www.pnas.org/cgi/doi/10.1073/pnas.0702538104>.

Kotsyfakis, M. et al., 2006. Antiinflammatory and Immunosuppressive Activity of Sialostatin L, a Salivary Cystatin from the Tick *Ixodes scapularis* * and Jose. *Insect Biochemistry and Molecular Biology*, 281(36), pp.26298–26307.

Lafaye, A. et al., 2004. Profiling of sulfoconjugates in urine by using precursor ion and neutral loss scans in tandem mass spectrometry. Application to the investigation of heavy metal toxicity in rats. *Journal of Mass Spectrometry*, 39(6), pp.655–664. Available at: <http://doi.wiley.com/10.1002/jms.635>.

Lehmann, T. & Diabate, A., 2008. The molecular forms of *Anopheles gambiae*: a phenotypic perspective. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases*, 8(5), pp.737–46. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/18640289>.

Leung, J.Y. & Kim, W.Y., 2013. Stearoyl co-A desaturase 1 as a ccRCC therapeutic target: death by stress. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 19(12), pp.3111–3. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23709675>.

Li, Z. et al., 2008. The lysosomal-mitochondrial axis in free fatty acid-induced hepatic lipotoxicity. *Hepatology*, 47(5), pp.1495–1503. Available at: <http://doi.wiley.com/10.1002/hep.22183>.

Liu, J. et al., 2003. Phospholipid scramblase 3 controls mitochondrial structure, function, and apoptotic response. *Molecular cancer research : MCR*, 1(12), pp.892–902. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/14573790>.

Liu, K. et al., 2013. Impact of trehalose transporter knockdown on *Anopheles gambiae* stress adaptation and susceptibility to *Plasmodium falciparum* infection. *Proceedings of the National Academy of Sciences of the United States of America*, 110(43), pp.17504–9. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24101462>.

Liu, N., 2015. Insecticide resistance in mosquitoes: impact, mechanisms, and research directions. *Annual review of entomology*, 60, pp.537–59. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25564745>.

Liu, X., Strable, M.S. & Ntambi, J.M., 2011. Stearoyl CoA desaturase 1: role in cellular inflammation and stress. *Advances in nutrition (Bethesda, Md.)*, 2(1), pp.15–22. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22211186>.

Luyimbazi, D. et al., 2010. Rapamycin Regulates Stearoyl CoA Desaturase 1 Expression in

- Breast Cancer. *Molecular Cancer Therapeutics*, 9(10), pp.2770–2784. Available at: <http://mct.aacrjournals.org/cgi/doi/10.1158/1535-7163.MCT-09-0980>.
- Luyimbazi, D. et al., 2010. Rapamycin regulates stearoyl CoA desaturase 1 expression in breast cancer. *Molecular cancer therapeutics*, 9(10), pp.2770–84. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20876744>.
- Maheswary, N.P. et al., 1993. Incrimination of *Anopheles annularis* van der Wulp-1854 as an epidemic malaria vector in Bangladesh. *The Southeast Asian journal of tropical medicine and public health*, 24(4), pp.776–778.
- Maheswary, N.P. et al., 1994. Incrimination of *Anopheles vagus* Donitz, 1902 as an epidemic malaria vector in Bangladesh. *Indian journal of malariology*, 31(1), pp.35–38.
- Maheswary, N.P., Habib, M.A. & Elias, M., 1992. Incrimination of *Anopheles aconitus* Donitz as a vector of epidemic malaria in Bangladesh. *The Southeast Asian journal of tropical medicine and public health*, 23(4), pp.798–801.
- Malik, S., 2004. Impact of the Metabolic Syndrome on Mortality From Coronary Heart Disease, Cardiovascular Disease, and All Causes in United States Adults. *Circulation*, 110(10), pp.1245–1250. Available at: <http://circ.ahajournals.org/cgi/doi/10.1161/01.CIR.0000140677.20606.0E>.
- Manoharan, M. et al., 2013. Comparative Genomics of Odorant Binding Proteins in *Anopheles gambiae*, *Aedes aegypti*, and *Culex quinquefasciatus*. *Genome Biology and Evolution*, 5(1), pp.163–180. Available at: <http://gbe.oxfordjournals.org/cgi/doi/10.1093/gbe/evs131>.
- Marinotti, O. et al., 2005. Microarray analysis of genes showing variable expression following a blood meal in *Anopheles gambiae*. *Insect molecular biology*, 14(4), pp.365–73. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16033430>.
- Marinotti, O., de Brito, M. & Moreira, C.K., 1996. Apyrase and alpha-glucosidase in the salivary glands of *Aedes albopictus*. *Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology*, 113(4), pp.675–9. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8925436>.
- Mauvoisin, D. et al., 2013. Decreasing stearoyl-CoA desaturase-1 expression inhibits β -catenin signaling in breast cancer cells. *Cancer Science*, 104(1), pp.36–42. Available at: <http://doi.wiley.com/10.1111/cas.12032>.
- Mazzalupo, S. et al., 2015. Effective disposal of nitrogen waste in blood-fed *Aedes aegypti* mosquitoes requires alanine aminotransferase. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/26310269>.
- Meyer, J.M. et al., 2012. A “Genome-to-Lead” Approach for Insecticide Discovery: Pharmacological Characterization and Screening of *Aedes aegypti* D1-like Dopamine Receptors R. R. Dinglasan, ed. *PLoS Neglected Tropical Diseases*, 6(1), p.e1478. Available at: <http://dx.plos.org/10.1371/journal.pntd.0001478>.
- Minville-Walz, M. et al., 2010. Inhibition of Stearoyl-CoA Desaturase 1 Expression Induces CHOP-Dependent Cell Death in Human Cancer Cells M. Polymenis, ed. *PLoS ONE*, 5(12), p.e14363. Available at: <http://dx.plos.org/10.1371/journal.pone.0014363>.
- Mitchell, G.A. et al., 1998. HMG CoA Lyase Deficiency: Identification of Five Causal Point Mutations in Codons 41 and 42, Including a Frequent Saudi Arabian Mutation, R41Q.

- The American Journal of Human Genetics*, 62(2), pp.295–300. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0002929707634946>.
- Mittal, P.K., Biolarvicides in vector control: challenges and prospects. *Journal of vector borne diseases*, 40(1-2), pp.20–32. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15119068>.
- Miyazaki, M. et al., 2007. Hepatic stearyl-CoA desaturase-1 deficiency protects mice from carbohydrate-induced adiposity and hepatic steatosis. *Cell metabolism*, 6(6), pp.484–96. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/18054317>.
- Miyazaki, M. et al., 2003. Identification and characterization of murine SCD4, a novel heart-specific stearyl-CoA desaturase isoform regulated by leptin and dietary factors. *The Journal of biological chemistry*, 278(36), pp.33904–11. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12815040>.
- Mondal, D. et al., 2010. Enhanced case detection and improved diagnosis of PKDL in a Kala-azar-Endemic area of Bangladesh. *PLoS Neglected Tropical Diseases*, 4(10), pp.1–8.
- Moreno-García, M. et al., 2014. Injury and immune response: applying the danger theory to mosquitoes. *Frontiers in Plant Science*, 5. Available at: <http://journal.frontiersin.org/article/10.3389/fpls.2014.00451/abstract>.
- Mostow, W.M. & Foster, W.A., 2004. Antagonistic effects of energy status on meal size and egg-batch size of *Aedes aegypti* (Diptera: Culicidae). *Journal of vector ecology: journal of the Society for Vector Ecology*, 29(1), pp.84–93.
- Müller, H.M. et al., 1995. Constitutive and blood meal-induced trypsin genes in *Anopheles gambiae*. *Experimental parasitology*, 81(3), pp.371–85. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/7498434>.
- Müller, H.M. et al., 1993. Members of a trypsin gene family in *Anopheles gambiae* are induced in the gut by blood meal. *The EMBO journal*, 12(7), pp.2891–900. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8335004>.
- Muoio, D.M. & Newgard, C.B., 2008. Fatty Acid Oxidation and Insulin Action: When Less Is More. *Diabetes*, 57(6), pp.1455–1456. Available at: <http://diabetes.diabetesjournals.org/cgi/doi/10.2337/db08-0281>.
- Narendra, D. et al., 2008. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *The Journal of Cell Biology*, 183(5), pp.795–803. Available at: <http://www.jcb.org/cgi/doi/10.1083/jcb.200809125>.
- Nguyen, L.N. et al., 2014. Stearyl Coenzyme A Desaturase 1 Is Associated with Hepatitis C Virus Replication Complex and Regulates Viral Replication. *Journal of Virology*, 88(21), pp.12311–12325. Available at: <http://jvi.asm.org/cgi/doi/10.1128/JVI.01678-14>.
- Nguyen, L.N. et al., 2014. Stearyl coenzyme A desaturase 1 is associated with hepatitis C virus replication complex and regulates viral replication. *Journal of virology*, 88(21), pp.12311–25. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25122791>.
- Nixon, J.E. et al., 1977. Metabolism and tissue distribution of label from [9,10-methylene-¹⁴C]sterculic acid in the rat. *Lipids*, 12(8), pp.629–34. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/895412>.
- Nsango, S.E. et al., 2013. AP-1/Fos-TGase2 Axis Mediates Wounding-induced *Plasmodium falciparum* Killing in *Anopheles gambiae*. *Journal of Biological Chemistry*, 288(22),

- pp.16145–16154. Available at: <http://www.jbc.org/cgi/doi/10.1074/jbc.M112.443267>.
- Ntambi, J.M., 1999. Regulation of stearyl-CoA desaturase by polyunsaturated fatty acids and cholesterol. *Journal of lipid research*, 40(9), pp.1549–1558.
- Ntambi, J.M. & Miyazaki, M., 2004. Regulation of stearyl-CoA desaturases and role in metabolism. *Progress in Lipid Research*, 43(2), pp.91–104.
- Omoti, C.E. et al., 2013. Prevalence of parasitemia and associated immunodeficiency among HIV-malaria co-infected adult patients with highly active antiretroviral therapy. *Asian Pacific journal of tropical medicine*, 6(2), pp.126–30. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23339914>.
- Ortinau, L.C. et al., 2012. Sterculic Oil, a Natural SCD1 Inhibitor, Improves Glucose Tolerance in Obese ob/ob Mice. *ISRN Endocrinology*, 2012, pp.1–11. Available at: <http://www.hindawi.com/journals/isrn/2012/947323/>.
- Ostrea, E.M. et al., 1985. Red cell membrane lipid peroxidation and hemolysis secondary to phototherapy. *Acta paediatrica Scandinavica*, 74(3), pp.378–81. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/4003061>.
- Park, E.-J. et al., 2014. Multiple pathways are involved in palmitic acid-induced toxicity. *Food and Chemical Toxicology*, 67, pp.26–34. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0278691514000416>.
- Payne, D., 1988. Use and limitations of light microscopy for diagnosing malaria at the primary health care level. *Bulletin of the World Health Organization*, 66(5), pp.621–626.
- Piotrowski, A. et al., 2006. Microarray-based survey of CpG islands identifies concurrent hyper- and hypomethylation patterns in tissues derived from patients with breast cancer. *Genes, chromosomes & cancer*, 45(7), pp.656–67. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16575877>.
- Plumb, R.S. et al., 2003. Use of liquid chromatography/time-of-flight mass spectrometry and multivariate statistical analysis shows promise for the detection of drug metabolites in biological fluids. *Rapid Communications in Mass Spectrometry*, 17(23), pp.2632–2638. Available at: <http://doi.wiley.com/10.1002/rcm.1250>.
- Povelones, M. et al., 2011. Structure-Function Analysis of the Anopheles gambiae LRIM1/APL1C Complex and its Interaction with Complement C3-Like Protein TEP1 D. S. Schneider, ed. *PLoS Pathogens*, 7(4), p.e1002023. Available at: <http://dx.plos.org/10.1371/journal.ppat.1002023>.
- Rahman, K.M. et al., 2010. Increasing incidence of post-kala-azar dermal leishmaniasis in a population-based study in Bangladesh. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 50(1), pp.73–76.
- Ranson, H., Collins, F. & Hemingway, J., 1998. The role of alternative mRNA splicing in generating heterogeneity within the Anopheles gambiae class I glutathione S-transferase family. *Proceedings of the National Academy of Sciences of the United States of America*, 95(24), pp.14284–9. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/9826692>.
- Reidenbach, K.R. et al., 2012. Patterns of genomic differentiation between ecologically differentiated M and S forms of Anopheles gambiae in West and Central Africa. *Genome biology and evolution*, 4(12), pp.1202–12. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23132896>.

- Reuben, R. et al., 1992. Mosquito blood feeding patterns as a factor in the epidemiology of Japanese encephalitis in southern India. *The American journal of tropical medicine and hygiene*, 46(6), pp.654–63. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/1320343>.
- Roelofs, W.L. & Rooney, A.P., 2003. Molecular genetics and evolution of pheromone biosynthesis in Lepidoptera. *Proceedings of the National Academy of Sciences of the United States of America*, 100(Suppl 2), p.14599. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16576755>.
- von Roemeling, C.A. et al., 2013. Stearoyl-CoA desaturase 1 is a novel molecular therapeutic target for clear cell renal cell carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 19(9), pp.2368–80. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23633458>.
- Rohde, J., Heitman, J. & Cardenas, M.E., 2001. The TOR kinases link nutrient sensing to cell growth. *The Journal of biological chemistry*, 276(13), pp.9583–6. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11266435>.
- Roongta, U. V. et al., 2011. Cancer Cell Dependence on Unsaturated Fatty Acids Implicates Stearoyl-CoA Desaturase as a Target for Cancer Therapy. *Molecular Cancer Research*, 9(11), pp.1551–1561. Available at: <http://mcr.aacrjournals.org/cgi/doi/10.1158/1541-7786.MCR-11-0126>.
- Scaglia, N., Chisholm, J.W. & Igal, R.A., 2009. Inhibition of StearoylCoA Desaturase-1 Inactivates Acetyl-CoA Carboxylase and Impairs Proliferation in Cancer Cells: Role of AMPK M. Bonini, ed. *PLoS ONE*, 4(8), p.e6812. Available at: <http://dx.plos.org/10.1371/journal.pone.0006812>.
- Scaglia, N., Chisholm, J.W. & Igal, R.A., 2009. Inhibition of stearoylCoA desaturase-1 inactivates acetyl-CoA carboxylase and impairs proliferation in cancer cells: role of AMPK. *PloS one*, 4(8), p.e6812. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19710915>.
- Schnitger, A.K.D. et al., 2009. Two C-type lectins cooperate to defend *Anopheles gambiae* against Gram-negative bacteria. *The Journal of biological chemistry*, 284(26), pp.17616–24. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19380589>.
- Schnitger, A.K.D., Kafatos, F.C. & Osta, M.A., 2007. The Melanization Reaction Is Not Required for Survival of *Anopheles gambiae* Mosquitoes after Bacterial Infections. *Journal of Biological Chemistry*, 282(30), pp.21884–21888. Available at: <http://www.jbc.org/cgi/doi/10.1074/jbc.M701635200>.
- Schnitger, A.K.D., Kafatos, F.C. & Osta, M.A., 2007. The melanization reaction is not required for survival of *Anopheles gambiae* mosquitoes after bacterial infections. *The Journal of biological chemistry*, 282(30), pp.21884–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17537726>.
- Scholte, E.-J. et al., 2005. An entomopathogenic fungus for control of adult African malaria mosquitoes. *Science (New York, N.Y.)*, 308(5728), pp.1641–2. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15947190>.
- Shahid, S., 2012. Vulnerability of the power sector of Bangladesh to climate change and extreme weather events. *Regional Environmental Change*, 12(3), pp.595–606. Available at: <http://link.springer.com/10.1007/s10113-011-0276-z>.
- Shanklin, J., Whittle, E. & Fox, B.G., 1994. Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-CoA desaturase, and are conserved

- in alkane hydroxylase and xylene monooxygenase. *Biochemistry*, 33(43), pp.12787–94. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/7947684>.
- SHANTZ, L.M., 2004. Transcriptional and translational control of ornithine decarboxylase during Ras transformation. *Biochemical Journal*, 377(1), pp.257–264. Available at: <http://biochemj.org/lookup/doi/10.1042/bj20030778>.
- Shapiro, J.P., Law, J.H. & Wells, M.A., 1988. Lipid transport in insects. *Annual review of entomology*, 33, pp.297–318. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/3277529>.
- Sinka, M.E. et al., 2011. The dominant Anopheles vectors of human malaria in the Asia-Pacific region: occurrence data, distribution maps and bionomic précis. *Parasites & Vectors*, 4(1), p.89. Available at: <http://www.parasitesandvectors.com/content/4/1/89>.
- Sodja, A., Fujioka, H., Lemos, F.J.A., et al., 2007. Induction of actin gene expression in the mosquito midgut by blood ingestion correlates with striking changes of cell shape. *Journal of Insect Physiology*, 53(8), pp.833–839. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0022191007000832>.
- Sodja, A., Fujioka, H., Lemos, F.J.A., et al., 2007. Induction of actin gene expression in the mosquito midgut by blood ingestion correlates with striking changes of cell shape. *Journal of insect physiology*, 53(8), pp.833–9. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17537455>.
- Soulages, J.L. & Wells, M.A., 1994. Lipophorin: the structure of an insect lipoprotein and its role in lipid transport in insects. *Advances in protein chemistry*, 45, pp.371–415. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8154373>.
- Stathopoulos, S. et al., 2014. Genetic Dissection of Anopheles gambiae Gut Epithelial Responses to Serratia marcescens D. S. Schneider, ed. *PLoS Pathogens*, 10(3), p.e1003897. Available at: <http://dx.plos.org/10.1371/journal.ppat.1003897>.
- Sun, J. et al., 2000. Lipophorin as a yolk protein precursor in the mosquito, Aedes aegypti. *Insect Biochemistry and Molecular Biology*, 30(12), pp.1161–1171. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S096517480000093X>.
- Surholt, B. et al., 1992. Insect lipophorin conversions. Compositional analysis of high- and low-density lipophorin of Acherontia atropos and Locusta migratoria. *Biological chemistry Hoppe-Seyler*, 373(1), pp.13–20. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/1536689>.
- Svegliati-Baroni, G. et al., 2006. A Model of Insulin Resistance and Nonalcoholic Steatohepatitis in Rats. *The American Journal of Pathology*, 169(3), pp.846–860. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0002944010627630>.
- Tarca, A.L., Romero, R. & Draghici, S., 2006. Analysis of microarray experiments of gene expression profiling. *American Journal of Obstetrics and Gynecology*, 195(2), pp.373–388. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0002937806008738>.
- Tauchman, S.J. et al., 2007. Effects of stress on the hemolymph juvenile hormone binding protein titers of Manduca sexta. *Insect Biochemistry and Molecular Biology*, 37(8), pp.847–854. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0965174807001130>.
- Thiberville, S.-D. et al., 2013. Chikungunya fever: epidemiology, clinical syndrome, pathogenesis and therapy. *Antiviral research*, 99(3), pp.345–70. Available at:

- <http://www.ncbi.nlm.nih.gov/pubmed/23811281>.
- Timson, D.J., 2007. Galactose Metabolism in *Saccharomyces cerevisiae*. *Dynamic Biochemistry, Process Biotechnology and Molecular Biology*, pp.63–73.
- della Torre, A., Tu, Z. & Petrarca, V., 2005. On the distribution and genetic differentiation of *Anopheles gambiae* s.s. molecular forms. *Insect Biochemistry and Molecular Biology*, 35(7), pp.755–769. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0965174805000597>.
- Trevino, V. & Falciani, F., 2007. DNA Microarrays: a Powerful Genomic Tool for Biomedical and Clinical Research. *Molecular Medicine*, 13(9-10), p.1. Available at: <http://www.molmed.org/content/pdfstore/527-541.Trevino.00107.PDF>.
- Uk, R.S.K., 2007. *Human health Coordinating Lead Authors : Lead Authors : Contributing Authors : Review Editors :*
- Varmus, H. et al., 2003. Public health. Grand Challenges in Global Health. *Science (New York, N.Y.)*, 302(5644), pp.398–399.
- VERMA, J.P., NATH, B. & AGGARWAL, J.S., 1955. Structure of Sterculic Acid. *Nature*, 175(4445), pp.84–85. Available at: <http://www.nature.com/doi/10.1038/175084a0>.
- Visintin, A. et al., 2006. MD-2. *Immunobiology*, 211(6-8), pp.437–47. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16920483>.
- Vlachou, D. et al., 2005. Functional Genomic Analysis of Midgut Epithelial Responses in *Anopheles* during Plasmodium Invasion. *Current Biology*, 15(13), pp.1185–1195. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0960982205006792>.
- Vogel, K.J., Brown, M.R. & Strand, M.R., 2015. Ovary ecdysteroidogenic hormone requires a receptor tyrosine kinase to activate egg formation in the mosquito *Aedes aegypti*. *Proceedings of the National Academy of Sciences of the United States of America*, 112(16), pp.5057–62. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25848040>.
- Volz, J., 2005. The Roles of Two Clip Domain Serine Proteases in Innate Immune Responses of the Malaria Vector *Anopheles gambiae*. *Journal of Biological Chemistry*, 280(48), pp.40161–40168. Available at: <http://www.jbc.org/cgi/doi/10.1074/jbc.M506191200>.
- Wältermann, M. & Steinbüchel, A., 2000. In vitro effects of sterculic acid on lipid biosynthesis in *Rhodococcus opacus* strain PD630 and isolation of mutants defective in fatty acid desaturation. *FEMS Microbiology Letters*, 190(1), pp.45–50. Available at: <http://femsle.oxfordjournals.org/cgi/doi/10.1111/j.1574-6968.2000.tb09260.x>.
- Wagatsuma, Y. et al., 2004. Dengue Fever Outbreak in a Recreation Club, Dhaka, Bangladesh. *Emerging Infectious Diseases*, 10(4), pp.747–750.
- Waldock, J., Olson, K.E. & Christophides, G.K., 2012. *Anopheles gambiae* Antiviral Immune Response to Systemic O'nyong-nyong Infection Y. M. Traub-Csekö, ed. *PLoS Neglected Tropical Diseases*, 6(3), p.e1565. Available at: <http://dx.plos.org/10.1371/journal.pntd.0001565>.
- Wältermann, M. & Steinbüchel, A., 2000. In vitro effects of sterculic acid on lipid biosynthesis in *Rhodococcus opacus* strain PD630 and isolation of mutants defective in fatty acid desaturation. *FEMS microbiology letters*, 190(1), pp.45–50. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10981688>.

- Wang, D. et al., 2002. Microarray-based detection and genotyping of viral pathogens. *Proceedings of the National Academy of Sciences of the United States of America*, 99(24), pp.15687–92. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12429852>.
- Warr, E. et al., 2006. Anopheles gambiae immune responses to Sephadex beads: Involvement of anti-Plasmodium factors in regulating melanization. *Insect Biochemistry and Molecular Biology*, 36, pp.769–778.
- Wickramage, K. et al., 2013. High attack rate for malaria through irregular migration routes to a country on verge of elimination. *Malaria journal*, 12(1), p.276. Available at: <http://www.malariajournal.com/content/12/1/276>.
- Win TT, Lin K, Mizuno S, et al., 2002. Wide distribution of Plasmodium ovale in Myanmar. *Trop Med Int Health*, 7, pp.231–9.
- Wolfender, J.-L. et al., 2015. Current approaches and challenges for the metabolite profiling of complex natural extracts. *Journal of Chromatography A*, 1382, pp.136–164. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0021967314017038>.
- Wolfender, J.-L. et al., 2009. MS-based plant metabolomic approaches for biomarker discovery. *Natural product communications*, 4(10), pp.1417–30. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19911581>.
- World Health Organization, 2014. A global brief on vector-borne diseases. *World Health Organization*, p.9. Available at: http://apps.who.int/iris/bitstream/10665/111008/1/WHO_DCO_WHD_2014.1_eng.pdf.
- Wu, Q. & Brown, M.R., 2006. Signaling and function of insulin-like peptides in insects. *Annual review of entomology*, 51, pp.1–24. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16332201>.
- Xia, Y.-Q. et al., 2003. Use of a quadrupole linear ion trap mass spectrometer in metabolite identification and bioanalysis. *Rapid Communications in Mass Spectrometry*, 17(11), pp.1137–1145. Available at: <http://doi.wiley.com/10.1002/rcm.1037>.
- Xu, W., Cornel, A.J. & Leal, W.S., 2010. Odorant-Binding Proteins of the Malaria Mosquito Anopheles funestus sensu stricto A. Hofmann, ed. *PLoS ONE*, 5(10), p.e15403. Available at: <http://dx.plos.org/10.1371/journal.pone.0015403>.
- Yamey G, T.E., 2002. The world's most neglected diseases [editorial]. *British Medical Journal*, 325, pp.176–177.
- Yang, Y., 2001. Role of Glutathione S-Transferases in Protection against Lipid Peroxidation. OVEREXPRESSION OF hGSTA2-2 IN K562 CELLS PROTECTS AGAINST HYDROGEN PEROXIDE-INDUCED APOPTOSIS AND INHIBITS JNK AND CASPASE 3 ACTIVATION. *Journal of Biological Chemistry*, 276(22), pp.19220–19230. Available at: <http://www.jbc.org/cgi/doi/10.1074/jbc.M100551200>.
- Yassine, H., Kamareddine, L. & Osta, M.A., 2012. The Mosquito Melanization Response Is Implicated in Defense against the Entomopathogenic Fungus Beauveria bassiana D. S. Schneider, ed. *PLoS Pathogens*, 8(11), p.e1003029. Available at: <http://dx.plos.org/10.1371/journal.ppat.1003029>.
- Yassine, H. & Osta, M.A., 2010. Anopheles gambiae innate immunity. *Cellular Microbiology*, 12(1), pp.1–9. Available at: <http://doi.wiley.com/10.1111/j.1462-5822.2009.01388.x>.
- Zhang, Y. et al., 2012. Poster: De novo expression fragment assembly from Spanning Trees (EAST). In *2012 IEEE 2nd International Conference on Computational Advances in Bio*

- and medical Sciences (ICCABS)*. IEEE, pp. 1–1. Available at:
<http://ieeexplore.ieee.org/lpdocs/epic03/wrapper.htm?arnumber=6182662>.
- Zhou, G., Flowers, M., et al., 2004. Metabolic fate of [14C]-labeled meal protein amino acids in *Aedes aegypti* mosquitoes. *Journal of insect physiology*, 50(4), pp.337–49. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15081827>.
- Zhou, G., Pennington, J.E. & Wells, M.A., 2004. Utilization of pre-existing energy stores of female *Aedes aegypti* mosquitoes during the first gonotrophic cycle. *Insect biochemistry and molecular biology*, 34(9), pp.919–25. Available at:
<http://www.ncbi.nlm.nih.gov/pubmed/15350611>.
- Zhu, J., Chen, L. & Raikhel, A.S., 2003. Posttranscriptional control of the competence factor FTZ-F1 by juvenile hormone in the mosquito *Aedes aegypti*. *Proceedings of the National Academy of Sciences*, 100(23), pp.13338–13343. Available at:
<http://www.pnas.org/cgi/doi/10.1073/pnas.2234416100>.
- Zieler, H. & Dvorak, J.A., 2000. Invasion in vitro of mosquito midgut cells by the malaria parasite proceeds by a conserved mechanism and results in death of the invaded midgut cells. *Proceedings of the National Academy of Sciences of the United States of America*, 97(21), pp.11516–21. Available at:
<http://www.ncbi.nlm.nih.gov/pubmed/11027351>.
- Zoeller, R.A. & Wood, R., 1985. The importance of the stearyl-CoA desaturase system in octadecenoate metabolism in the Morris hepatoma 7288C. *Biochimica et biophysica acta*, 845(3), pp.380–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/2860925>.
- Zofou, D. et al., 2014. Control of malaria and other vector-borne protozoan diseases in the tropics: enduring challenges despite considerable progress and achievements. *Infectious Diseases of Poverty*, 3(1), p.1. Available at:
<http://www.idpjournals.com/content/3/1/1>.
- Zolotukhin, A.S. et al., 2009. Nuclear export factor RBM15 facilitates the access of DBP5 to mRNA. *Nucleic Acids Research*, 37(21), pp.7151–7162. Available at:
<http://nar.oxfordjournals.org/lookup/doi/10.1093/nar/gkp782>.

Chapter 9

Appendix

Table:S1 List of genes which show altered expression across time points in SCD1 KD mosquitoes

Transcript ID	Functional Classification	Name of the gene	Fold changes				
			0h	6h	12h	18h	24h
AGAP001942-RA	Carbohydrate and Lipid metabolism	Fumarylacetoacetase, C-terminal-related	-1.49	-1.5	-1.94965	-2.1	-2.07
AGAP004352-RB	Carbohydrate and Lipid metabolism	succinyl-CoA synthetase beta subunit	-2.06	-2.4	-1.81	-1.4	-1.6
AGAP001713-RB	Carbohydrate and Lipid metabolism	stearoyl-CoA desaturase (delta-9 desaturase)	-2.98	-5.78	-2.9	-1.49	-1.52
AGAP009463-RA	Carbohydrate and Lipid metabolism	ATP-binding cassette transporter (ABC transporter) family G member	-1.31	1.057997	-1.68	-2.46	-1.43
AGAP008717-RA	Carbohydrate and Lipid metabolism	hydroxymethylglutaryl-CoA lyase	-1.57	-2	-1.33	-1.298	-1.43
AGAP001713-RC	Carbohydrate and Lipid metabolism	stearoyl-CoA desaturase (delta-9 desaturase)	-3.22	-4.5	-2.73	-1.3	-1.4
AGAP004352-RA	Carbohydrate and Lipid metabolism	succinyl-CoA synthetase beta subunit	-1.92	-2.37	-1.51	-1.45	-1.3
AGAP006775-RA	Carbohydrate and Lipid metabolism	Glucosyl/glucuronosyl transferases	-1.18	-1.21	-2	-1.45401	-1.3
AGAP006740-RA	Carbohydrate and Lipid metabolism	dolichyl-phosphate mannosyltransferase polypeptide 2, regulatory subunit	-2.01	-3.96	-2.12	-1.24	-1.26
AGAP001200-RB	Carbohydrate and Lipid metabolism	glycogen debranching enzyme	-1.23	-2.2	-2.46	-1.298	-1.119
AGAP001200-RA	Carbohydrate and Lipid metabolism	glycogen debranching enzyme	-1.11	-1.5	-2.24	-1.1	-1.03
AGAP003049-RC	Carbohydrate and Lipid metabolism	stearoyl-CoA desaturase (delta-9 desaturase)	-1.36	-2.35	-1.52	-1.27	-1.03
AGAP011984-RA	Carbohydrate and Lipid metabolism	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 20	-1.33	1.340404	-1.11	2.079835	1.034114
AGAP005504-RA	Carbohydrate and Lipid metabolism	phospholipid scramblase 1	1.069448	2.220635	2.10041	1.914016	1.472575
AGAP005504-RD	Carbohydrate and Lipid metabolism	phospholipid scramblase 1	1.126298	2.035964	1.78991	1.873443	1.691984
AGAP005504-RB	Carbohydrate and Lipid metabolism	phospholipid scramblase 1	1.210008	2.226592	1.755443	2.110448	1.694627

AGAP010695-RA	Carbohydrate and Lipid metabolism	elongation of very long chain fatty acids protein 4	1.206646	-1.1	-1.15	-1.4	2.084245
AGAP011948-RA	Carbohydrate and Lipid metabolism	threonine 3-dehydrogenase	-1.05	-1.409	1.178857	1.571312	2.291269
AGAP002032-RA	Carbohydrate and Lipid metabolism	Lipid transport protein	1.507056	1.350371	1.431939	2.067538	2.704781
AGAP004426-RB	Cytoskeleton / Cell adhesion / Structural components	FAS1 domain	1.087899	1.050508	-1.278	-1.43834	-2.03
AGAP005360-RA	Cytoskeleton / Cell adhesion / Structural components	PQ loop repeat-containing protein 3	-1.054	1.009671	-2.3	-1.26	1.191178
AGAP005095-RA	Cytoskeleton / Cell adhesion / Structural components	actin beta/gamma 1	-1.08	2.042297	1.067581	-1.27	1.296873
AGAP004935-RA	Cytoskeleton / Cell adhesion / Structural components	Ankyrin repeat	1.050927	2.277828	1.375176	1.620239	1.355118
AGAP006148-RA	Cytoskeleton / Cell adhesion / Structural components	CPLCA3	1.018452	-1.17	1.904501	2.109506	1.376727
AGAP009200-RB	Cytoskeleton / Cell adhesion / Structural components	collagen, type IV, alpha	1.823213	1.4458	1.501694	1.579754	2.103669
AGAP000969-RB	Cytoskeleton / Cell adhesion / Structural components	tropomodulin	1.212274	1.396145	-1.3	1.394434	2.262444
AGAP007103-RD	Cytoskeleton / Cell adhesion / Structural components	Calsyntenin-1	-1	1.233959	1.334675	1.479464	2.285695
AGAP001381-RA	Cytoskeleton / Cell adhesion / Structural components	laminin, beta 1	1.398535	1.655494	1.097785	1.266421	2.349416
AGAP009790-RA	Cytoskeleton / Cell adhesion / Structural components	CPAP3	1.511569	1.113605	2.099975	1.434819	3.256924

AGAP006647-RB	Immunity / Putative immunity / Phagocytosis	Leucine-rich repeat domain	-1.22	1.063556	-1.077	-1.067	-2.08
AGAP006647-RA	Immunity / Putative immunity / Phagocytosis	Leucine-rich repeat domain	-1.32	-1.04	-1.11	-1.14	-2.07
AGAP008654-RA	Immunity / Putative immunity / Phagocytosis	TEP12	-1.3	-1.181	-2	-1.256	-1.2
AGAP001769-RB	Immunity / Putative immunity / Phagocytosis	Beat protein	1.688098	1.483634	1.836994	2.156669	1.132659
AGAP006909-RA	Immunity / Putative immunity / Phagocytosis	SPRN1	1.473078	2.235072	1.560846	1.372	1.160503
AGAP001769-RD	Immunity / Putative immunity / Phagocytosis	Beat protein	1.978516	2.083454	1.952135	2.104706	1.163552
AGAP001769-RC	Immunity / Putative immunity / Phagocytosis	Beat protein	1.764019	2.100596	1.508109	2.080967	1.311774
AGAP000720-RB	Cytoskeleton / Cell adhesion / Structural components	Neuronal cell adhesion molecule	2.678655	1.102464	1.210577	-1.03	1.360428
AGAP005246-RE	Immunity / Putative immunity / Phagocytosis	SRPN10	-1.06	2.150949	-1.1	1.348671	1.439932
AGAP009215-RA	Immunity / Putative immunity / Phagocytosis	CLIPB18	1.526035	1.814823	1.287792	1.776654	2.179525
AGAP003610-RA	Immunity / Putative immunity / Phagocytosis	Immunoglobulin I-set	1.902506	1.696711	2.173595	1.712431	2.260365
AGAP007209-RB	Immunity / Putative immunity / Phagocytosis	Tetraspanin	1.689261	2.275483	1.271376	1.701983	2.336936

AGAP002422-RA	Immunity / Putative immunity / Phagocytosis	CLIPD1	1.398917	1.37124	1.585267	1.802481	2.566489
AGAP008927-RA	Immunity / Putative immunity / Phagocytosis	protein TILB homolog	-1	1.020153	1.340146	3.754717	3.16982
AGAP012757-RA	Protein degradation / Proteasome	Aminopeptidase N1	-2.89	-1	-1.47	-1.788	-3.97
AGAP000901-RA	Protein degradation / Proteasome	Alanine transaminase	-2.2	-1.44	-1.54	-1.987	-2.35
AGAP002720-RA	Protein degradation / Proteasome	Cathepsin O	-2.05	-1.347	-1.38	-1.4	-1
AGAP012662-RA	Protein degradation / Proteasome	Omega-amidase	-1.6	-1.63	-2.048	-1.11	-1.052
AGAP009266-RA	Protein degradation / Proteasome	Low molecular weight phosphotyrosine protein phosphatase	-1.07	1.256108	-1.14	1.13554	2.086455
AGAP002878-RA	Protein degradation / Proteasome	Cystatin-like protein	1.491348	2.055883	1.006411	1.56786	2.23069
AGAP011909-RA	Protein degradation / Proteasome	Peptidase S1	1.199041	1.164367	1.785166	1.593683	2.449878
AGAP010885-RA	Redox / Apoptosis / Detoxification	(S)-2-hydroxy-acid oxidase	-1.67032	-1.07963	-1.80772	-2.1667	-2.41845
AGAP003785-RD	Redox / Apoptosis / Detoxification	glucose dehydrogenase (acceptor)	-1.48791	-1.03926	-1.33031	-1.34718	-2.40031
AGAP006023-RA	Redox / Apoptosis / Detoxification	tyrosine 3-monooxygenase	-1.00436	-1.10172	1.001112	-1.3767	-2.025
AGAP005009-RA	Redox / Apoptosis / Detoxification	pyrroline-5-carboxylate reductase	-2.15985	-1.33513	-1.6371	-1.11284	-1.37597
AGAP000109-RA	Redox / Apoptosis / Detoxification	cytochrome c oxidase subunit VIIa	-1.24869	-2.38858	-1.51732	-1.27944	-1.359
AGAP003167-RA	Redox / Apoptosis / Detoxification	NAD(P) transhydrogenase	-2.21316	-1.93682	-1.79971	-1.76453	-1.22634
AGAP011507-RA	Redox / Apoptosis / Detoxification	COE13O	-2.70626	-2.35412	-2.71888	-1.37181	1.031038

	n						
AGAP009783-RA	Redox / Apoptosis / Detoxification	short/branched chain acyl-CoA dehydrogenase	-1.20807	-2.05442	-1.5835	-1.11933	1.202935
AGAP011334-RA	Redox / Apoptosis / Detoxification	Failed axon connections protein	1.265732	2.36361	1.599421	1.444883	1.485407
AGAP000327-RA	Redox / Apoptosis / Detoxification	tyrosine aminotransferase	1.167939	1.457156	1.082345	1.592865	2.089807
AGAP011066-RA	Redox / Apoptosis / Detoxification	Aldose reductase	1.001711	1.085757	1.076431	1.182333	2.205956
AGAP004163-RB	Redox / Apoptosis / Detoxification	GSTD7	1.57039	1.707788	1.700157	3.093798	2.491309
AGAP004592-RI	Replication/ Transcription / Translation / Transcription factors / Cell cycle	splicing factor, arginine/serine-rich 4/5/6	-1.46409	-1.24769	-1.15852	-2.10307	-2.59803
AGAP004592-RA	Replication/ Transcription / Translation / Transcription factors / Cell cycle	splicing factor, arginine/serine-rich 4/5/6	-1.49885	-1.44273	-1.23606	-1.94727	-2.59297
AGAP005336-RA	Replication/ Transcription / Translation / Transcription factors / Cell cycle	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 1	-1.07713	-1.12366	-1.04774	-1.44314	-2.36242
AGAP005127-RA	Replication/ Transcription / Translation / Transcription factors / Cell cycle	RNA-binding protein 15	-1.14069	-1.15556	-1.00814	-1.3185	-2.22773
AGAP004592-RH	Replication/ Transcription / Translation / Transcription factors / Cell cycle	splicing factor, arginine/serine-rich 4/5/6	-1.58048	-1.09617	-1.03162	-1.74247	-2.22488
AGAP006702-RB	Replication/ Transcription / Translation / Transcription factors / Cell	No metadata available	-1.01439	1.037068	-1.48988	-1.06938	-2.06748

	cycle						
AGAP012413-RA	Replication/ Transcription / Translation / Transcription factors / Cell cycle	CycA	- 1.11569	1.09956 3	1.34126 3	-1.40038	- 2.04775
AGAP007967-RA	Replication/ Transcription / Translation / Transcription factors / Cell cycle	selenide, water dikinase	- 1.19435	-2.58662	1.05057 3	-1.68258	- 1.87734
AGAP005122-RA	Replication/ Transcription / Translation / Transcription factors / Cell cycle	UBX domain-containing protein 1	1.00940 2	1.00376 8	-1.3122	-2.32226	-1.5898
AGAP003912-RA	Replication/ Transcription / Translation / Transcription factors / Cell cycle	histone H2B	1.02260 5	-1.37349	2.03536 7	1.02038	- 1.29027
AGAP000179-RC	Replication/ Transcription / Translation / Transcription factors / Cell cycle	Amidophosphoribosyltransfer ase	- 1.45003	-3.98579	-2.08107	-1.19225	- 1.06942
AGAP000179-RB	Replication/ Transcription / Translation / Transcription factors / Cell cycle	Amidophosphoribosyltransfer ase	- 1.41135	-2.78225	-1.65886	-1.09555	1.06507
AGAP000179-RA	Replication/ Transcription / Translation / Transcription factors / Cell cycle	Amidophosphoribosyltransfer ase	- 1.42266	-3.9325	-1.66248	-1.13039	1.09270 6
AGAP001544-RA	Replication/ Transcription / Translation / Transcription factors / Cell cycle	tripartite motif-containing protein 37	1.42384 4	1.02008 3	2.02865 7	1.58834 1	1.17767 5
AGAP003913-RA	Replication/ Transcription / Translation / Transcription factors / Cell cycle	histone H2A	- 1.06758	-1.02007	-1.10408	1.15330 8	2.0182

AGAP007103-RB	Replication/ Transcription / Transcription factors / Cell cycle	Calsyntenin-1	- 1.06788	1.20135	1.2666	1.33152 5	2.03586 2
AGAP003911-RA	Replication/ Transcription / Transcription factors / Cell cycle	histone H2A	1.09366 2	1.01948 9	-1.19246	-1.05034	2.09430 4
AGAP002670-RB	Replication/ Transcription / Transcription factors / Cell cycle	Zinc finger, ZZ-type	1.63564 9	1.89716 6	1.74999	1.49287 6	2.18753 2
AGAP010600-RA	Signaling / ATPases / GTPases	Insulin-like peptide 2 precursor	- 1.23174	1.10728 2	-1.17156	-1.29054	-2.1499
AGAP003720-RA	Signaling / ATPases / GTPases	Annexin A4	1.00806 5	2.21156 9	1.66469 5	-1.05379	- 1.33895
AGAP005627-RC	Signaling / ATPases / GTPases	creatine kinase	- 1.06868	2.02503 8	1.24258 2	-1.23439	1.16527 1
AGAP000297-RA	Signaling / ATPases / GTPases	No metadata available	1.45773 9	2.40873 6	1.89918 2	1.88105	1.47547 1
AGAP000617-RB	Signaling / ATPases / GTPases	dual specificity phosphatase	1.18230 2	1.18840 6	1.67880 1	1.84419	2.01343 8
AGAP002905-RA	Signaling / ATPases / GTPases	OBP13	1.21523 4	-1.02322	1.08452 9	1.43363 5	2.04193 9
AGAP013136-RA	Signaling / ATPases / GTPases	ATPase	1.31706 1	1.60766 6	1.44228 8	1.28104 9	2.04839 9
AGAP002858-RC	Signaling / ATPases / GTPases	sodium/potassium- transporting ATPase subunit alpha	1.43029 9	2.14642 2	1.60371 5	1.35114 2	2.05708 6
AGAP005162-RB	Signaling / ATPases / GTPases	dystroglycan 1	2.26079 2	1.64294 1	1.92808 9	1.69152 2	2.06121 5
AGAP010225-RA	Signaling / ATPases / GTPases	ER lumen protein retaining receptor	1.47563 4	1.74316 1	-1.07569	1.28757 5	2.11494 9
AGAP003121-RA	Signaling / ATPases / GTPases	phosphatidylinositol 4-kinase type 2	1.70134 3	1.70188 3	1.84241 6	1.50684 5	2.23323 5
AGAP012755-RA	Signaling / ATPases / GTPases	ER lumen protein retaining receptor	1.45036 5	1.62505 4	-1.09839	1.47564 8	2.24045 5

AGAP012321-RA	Signaling / ATPases / GTPases	OBP26	-1.05546	1.131918	1.20807	1.1504	2.269414
AGAP001573-RA	Signaling / ATPases / GTPases	Ras-related C3 botulinum toxin substrate 1	1.567719	1.881858	1.714255	1.64289	2.286472
AGAP008054-RA	Signaling / ATPases / GTPases	chemosensory protein	-1.04478	-2.37925	1.433397	2.273071	2.410843
AGAP012302-RA	Transport / Vesicle mediated transport	Sodium-independent sulfate anion transporter	-1.57693	-1.05192	-2.29933	-1.04725	-1.63552
AGAP005563-RC	Transport / Vesicle mediated transport	Tret1	-2.10731	-1.09866	-1.68863	1.059881	-1.15982
AGAP010975-RA	Transport / Vesicle mediated transport	Sodium/potassium/calcium exchanger	-1.28861	-1.10975	-2.25794	-1.37641	-1.01364
AGAP004519-RB	Transport / Vesicle mediated transport	Sideroflexin 1,2,3	-1.43511	-2.57205	-1.32659	1.121886	1.078727
AGAP001487-RA	Transport / Vesicle mediated transport	innexin shaking-B	1.190948	1.138892	1.267057	2.038185	1.940026
AGAP011062-RA	Transport / Vesicle mediated transport	Hemoglobin (Heterodimeric)	1.595408	2.319121	1.482087	1.454539	1.956269
AGAP005933-RA	Transport / Vesicle mediated transport	NFkappaB essential modulator	1.287767	2.175518	1.765074	1.439133	2.016453
AGAP001550-RA	Transport / Vesicle mediated transport	Sodium-coupled monocarboxylate transporter 1	1.108476	1.059807	-1.09976	1.260383	2.030383
AGAP004896-RA	Transport / Vesicle mediated transport	potassium channel, subfamily K, member 2	1.356011	1.085395	1.024606	1.319575	2.044282
AGAP000128-RA	Transport / Vesicle mediated transport	MFS transporter, VNT family, synaptic vesicle glycoprotein 2	-1.14218	1.114404	1.074194	1.365641	2.393502
AGAP000242-RA	Transport / Vesicle mediated transport	vesicle transport protein SEC20	2.537641	1.647824	1.919043	1.498809	2.703308
AGAP006123-RA	Unknown	No metadata available	-1.30829	-1.22385	-2.03879	-2.14118	-3.53638
AGAP000867-RA	Unknown	No metadata available	-1.09293	1.079907	-1.13828	-1.32676	-2.11095

AGAP012609-RA	Unknown	No metadata available	- 1.20116	-1.4271	-1.38345	-1.99635	- 2.05029
AGAP005585-RC	Unknown	No metadata available	-1.4192	-1.26974	-2.1081	-1.51304	- 1.52844
AGAP001890-RA	Unknown	No metadata available	-2.1215	-1.03305	-1.54515	-1.36342	- 1.39432
AGAP003550-RA	Unknown	No metadata available	1.03296 1	-1.12179	2.07707 5	-1.15416	- 1.33663
AGAP000964-RA	Unknown	No metadata available	1.38356 9	2.04798 2	1.23891 2	1.48023 8	1.28698 6
AGAP005379-RA	Unknown	No metadata available	2.19132	1.60699 8	1.63187 1	1.31179 7	1.35577 5
AGAP001718-RA	Unknown	No metadata available	1.20576 8	2.11458 7	1.36283 6	1.41685 4	1.65515
AGAP003352-RC	Unknown	No metadata available	1.23862 1	2.08234 8	1.52309 7	1.45247 1	1.69994 1
AGAP013481-RA	Unknown	No metadata available	- 1.25197	2.07281	-1.30042	1.16863 5	1.80721 6
AGAP010045-RA	Unknown	No metadata available	1.77317 6	1.42534 2	2.02261 1	1.69274 7	1.89711
AGAP006971-RA	Unknown	No metadata available	2.00859 4	1.97102 2	1.57398 1	1.55104 4	1.95997 9
AGAP007185-RA	Unknown	No metadata available	1.66854 9	1.80358 9	1.52235 7	1.43400 2	2.01494
AGAP005813-RA	Unknown	No metadata available	1.14561 9	1.47620 6	1.3618	1.46248 9	2.03220 2
AGAP010148-RA	Unknown	No metadata available	1.94867 8	1.59348 7	1.39269	1.82905 9	2.13398 5
AGAP011044-RA	Unknown	No metadata available	1.12288 5	1.65632 5	-1.04336	1.64708 4	2.13851 2
AGAP002085-RC	Unknown	No metadata available	- 1.01175	1.75586	1.30576	1.83632 4	2.17971 4
AGAP000697-RA	Unknown	No metadata available	1.95320 4	1.31635 1	1.71575 7	1.30109 5	2.18972 8
AGAP002085-RA	Unknown	No metadata available	- 1.08349	1.70638	1.28402 7	1.38770 5	2.20917 5
AGAP002085-RB	Unknown	No metadata available	1.07831 4	1.41568 4	1.36859 2	1.61797 7	2.30857 6
AGAP013076-RA	Unknown	No metadata available	1.38092 9	1.66265 3	1.34446 7	1.19737 7	2.31247 5
AGAP010066-RA	Unknown	No metadata available	1.30003 5	2.23881 6	-1.13792	1.67577 1	2.56786 1
AGAP001174-RA	Unknown	No metadata available	2.43324 2	3.20621	2.72398	2.43877 1	2.64005 1
AGAP007842-RA	Unknown	No metadata available	1.86841 5	2.37360 7	2.60963	1.70077 3	2.66427 9
AGAP003060-RA	Unknown	No metadata available	2.49524 2	1.48991 5	2.53442 6	1.72857 1	2.78527 7

AGAP009462-RA	Unknown	No metadata available	2.199095	1.92223	1.781883	1.7207	3.328377
AGAP011576-RA	Unknown	No metadata available	2.691398	2.044409	2.013618	1.836891	3.430441
AGAP003773-RA	Unknown	No metadata available	1.333928	1.87219	-1.12195	2.026548	3.841509
AGAP007314-RA	Unknown	No metadata available	2.873416	1.75405	1.504276	3.147106	5.37099
AGAP011065-RA	Unknown	No metadata available	1.043243	-1.34945	1.603437	2.371093	5.633541

Table: S2 List of immune genes which show differential expression in *SCD1* KD mosquitoes

Gene ID	Name	Fold changes				
		0h	6h	12h	18h	24h
AGAP011787-RA	CLIPA5	4.637564	4.176203	No data	1.692576	3.608658
AGAP010731-RA	CLIPA8	1.060295	No data	1.328005	No data	2.295051
AGAP009214-RA	CLIPB11	1.415461	No data	No data	1.907089	2.248525
AGAP009217-RA	CLIPB12	4.878154	No data	2.448646	No data	2.879107
AGAP010833-RA	CLIPB14	3.052399	No data	1.656077	1.800905	2.373628
AGAP009844-RA	CLIPB15	1.834629	No data	No data	2.1827	3.402466
AGAP001648-RA	CLIPB17	5.261095	No data	4.61839	4.093173	No data
AGAP009215-RA	CLIPB18	1.526035	1.814823	1.287792	1.776655	2.179525
AGAP003247-RA	CLIPB19	1.018067	1.244041	No data	2.832054	1.736463
AGAP012037-RA	CLIPB20	1.223776	2.076342	No data	-1.09205	1.019822
AGAP002422-RA	CLIPD1	1.398917	1.37124	1.585267	1.802481	2.566488
AGAP007456-RA	LRRIM8B	No data	-2.49836	-2.36044	No data	No data
AGAP007455-RA	LRRIM10	-1.35984	No data	-2.78161	No data	No data
AGAP010816-RA	TEP3	2.456022	No data	1.9697	2.094799	4.355418
AGAP008654-RA	TEP12	1.311118	1.181009	2.007956	1.256317	1.203668
AGAP010811-RA	FREP19	No data	1.132828	1.739411	5.656849	No data
AGAP010763-RA	FREP21	No data	1.655453	1.065637	2.88555	3.027795
AGAP011228-RA	FREP24	No data	No data	4.051735	No data	No data
AGAP010759-RA	FREP32	No data	2.443725	1.668615	No data	2.673858
AGAP011231-RA	FREP59	No data	2.08607	1.018616	1.164993	1.435175
AGAP012352-RA	ML1	-1.85754	No data	-4.97349	No data	No data
AGAP002804-RA	ML4	No data	No data	No data	3.715127	No data
AGAP002849-RA	ML7	No data	No data	1.315672	9.261548	6.937465
AGAP004017-RA	Leucine rich protein	1.604855	No data	1.777525	2.573785	2.989334
AGAP006647-RA	Leucine rich protein	1.323321	1.046415	1.112405	1.149128	2.027941
AGAP000693-RA	CEC-1	3.640545	No data	2.455529	2.327022	4.479035
AGAP005334-RA	CTLMA2	2.149406	No data	1.483791	2.090924	2.619961
AGAP004247-RC	GPXH1	1.25162	2.064726	No data	1.618945	1.587142
AGAP005848-RA	Ficolin-A	1.106999	3.143801	1.788332	No data	1.365175

AGAP008179-RA	SCRBQ3	No data	No data	No data	2.314181	No data
AGAP004975-RA	PPO3	No data	No data	1.191027	1.026363	2.979984
AGAP009200-RB	Collagen IV	1.823213	1.4458	1.501695	1.579754	2.10367
AGAP007159-RA	Alpha-crystallin B chain	No data	No data	1.009312	1.3502	2.077811
AGAP008645-RA	GAM1	No data	1.291917	No data	1.762335	2.112557
AGAP001212-RA	PGRPLB1	No data	No data	No data	No data	2.3273
AGAP001212-RB	PGRLB2	No data	No data	No data	No data	2.237026
AGAP007209-RB	TETRASPANIN	1.689261	2.275483	1.271377	1.701983	2.336935
AGAP010759-RA	TETRASPANIN	No data	2.443725	1.668615	No data	2.673858