2. Review of literature
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2.1. Lymphatic filariasis

There are nine species of filarial parasitic nematodes infecting and causing the disease, filariasis, in human. They are *W. bancrofti*, *B. malayi*, *Brugia timori*, *Onchocerca volvulus*, *Loa loa*, *Mansonella ozzardi*, *Mansonella perstans*, *Mansonella streptocerca* and *Brugia pahangi*. Out of these, the later four species are less common unnatural infections contracted from animals. Adults of the filarial parasites are tissue specific in the human host and their embryonic stage, called microfilariae are found in the blood or skin of the host, depending upon the species of the parasite. The adults of *O. volvulus*, dwell in skin nodules and eye, and the mf are found in the skin. Those of *L. loa* dwell in the subcutaneous tissues with the presence of mf in the blood.

Adults of *W. bancrofti*, *B. malayi* and *B. timori* live in the lymphatic system, especially the lymphatic vessels and the nodes, and hence they cause lymphatic filariasis. The mf of these nematodes are sheathed, and are found in the blood. The major parasitic agents of lymphatic filariasis are *W. bancrofti* and *B. malayi* causing morbidity in over 100 million individuals world wide and the total population at risk is estimated to be 1.3 million (Bockarie et al., 1998; WHO, 2007). The disease caused by *W. bancrofti* alone accounts for 90% of the cases and India alone contributes about 40% of the total global burden of this disease (Ramaiah et al., 2000). This parasite is distributed throughout the tropical regions of Asia, Africa, China, the Pacific and isolated locations in the Americas. In India, *W. bancrofti* is widely distributed in 17 states and union territories and accounts for about 98% of the national burden. The endemic range of *B. malayi* is confined to South and South-East Asia from India in the west to Korea in the east. In India, this parasite is restricted to places like the coastal belts of Kerala, where the breeding grounds of the vector mosquitoes are plenty. *B. timori* is localized in Lesser Sunda Islands of Eastern Indonesia (WHO, 1992).
2.2. The filarial nematodes

Nematodes have long been considered as a Class under the Phylum Aschelminthes. However, since those characteristics used to show relationships among various classes in the Aschelminthes are now questionable, nematodes are now placed in a separate Phylum, Nematoda (Inglis, 1983). Under this Phylum, there are two Classes, the Secernentea (Phasmidia) and the Adenophorea (Aphasmidia). Both these Classes of Nematoda have several Orders with vertebrate parasitic worms (Anderson et al., 1983). Class Secernentea has 7 Orders, which include Ascaridida, containing the human intestinal ascaris worms, and Spirurida, containing the dracunculid guinea worm and various filarial worms. The lymphatic filarial parasites belong to the Phylum Nematoda, Class Secernentea, Order Spirurida, Suborder Spirurina, Superfamily Filaroidea, Family Onchocercidae, Subfamily Onchocercinae, and Genera Wuchereria/Brugia.

Since *W. bancrofti* and *B. malayi* co-exist in many places (Raina et al., 1990; Rajendran et al., 1997), their identification to species level is very important in diagnosis as well as epidemiological surveys. These species could be differentiated based on the measurements of various body characters of the mf, such as the length, width, cephalic space, length to width ratio, and position of nerve ring, excretory pore, innenkorper and anal pore, when compared to total body length. Among these, the most important body characters to differentiate *W. bancrofti* mf from *B. malayi* are the differences in the cephalic space and the number of caudal nuclei. The cephalic length to width ratio of *W. bancrofti* is 1:1 and that of *B. malayi* is 2:1. *W. bancrofti* has only one caudal nucleus whereas *B. malayi* has two caudal nuclei (Plate 1).

**Plate 1**: Microfilaria of *W. bancrofti* showing cephalic space (CS), nerve ring (NR), excretory pore (EP), and caudal nucleus (CN)
The infective third stage larvae (L3) of these species, by dissecting out from mosquito vectors, can be differentiated by examining the caudal papillae. There are three caudal papillae, two lateral and one terminal (Plate 2). All the three caudal papillae are distinctly protruding in *W. bancrofti* as compared to *B. malayi*. The lateral papillae of *B. malayi*, under electron microscopy, show a gutter-like indentation around their bases and this character is absent in *W. bancrofti* (Zaman and Narayanan, 1986).

Plate 2*: Caudal papillae of infective stage larva of *Wuchereria bancrofti*

2.2.1. Different strains of the parasite, based on the periodicity of microfilariae:

Three genetically determined physiological races exist in *W. bancrofti* and *B. malayi*, depending on the microfilarial periodicity (Sasa and Tanaka, 1974; Tanaka, 1981; Gupta *et al.*, 1990; Tewari *et al.*, 1995; Weerasooriya *et al.*, 1998; Shriram *et al.*, 2002; Pichon and Treuil, 2004). They are the nocturnally periodic, nocturnally sub-periodic, and diurnally sub-periodic forms (Plate 3). In the Indian sub-continent, both the parasites exist as nocturnally periodic forms and the mf appear in the peripheral circulation at night. Diurnally sub-periodic strain of *W. bancrofti* occurs in many islands in South Pacific, French Polynesia, Thailand and Nicobar islands. The mf remains in the peripheral blood all the time with a peak density at 12 to 20 h. The zoonotic *B. malayi* prevalent in Indonesia and Malaysia is nocturnally sub-periodic and the mf is present in the peripheral blood at all times, with a slight nocturnal rise. The *B. timori* parasite present in the Timor Island is nocturnally periodic.
Plate 3: Different strains of lymphatic filarial parasites based on periodicity of microfilariae, and its geographic distribution.

Various hypotheses have been postulated on the mechanism of microfilarial periodicity. As early as in 1951, Hawking and Thurston demonstrated that periodic fluctuation in the number of mf was due to their accumulation in the lungs during daytime and release to the circulating blood at night. Stimuli like body temperature are shown to have effect on the periodicity of mf of *W. bancrofti*, *B. malayi*, and *Dirofilaria repens* (Hawking et al., 1966). Later, it was postulated that accumulation of mf in lungs is due to greater oxygen in the lungs during the day compared to that in the night when the host is under rest (Hawking and Clark, 1967; Hawking et al., 1981). However, why and how mf remains in lungs during the day and the exact mechanism of mf periodicity as such is not yet fully understood. The periodicity of mf seems to be oriented to the 24 h cycle of the host in which the circadian rhythm of the host could act as a cue to the control of the circadian rhythm of the mf. Whatever be the reason, the mf periodicity is in agreement with the feeding behaviour of the vector mosquito (Vanamail and Ramaiah, 1991; Weerasooriya et al., 1998), enabling the mosquito to ingest the mf in large numbers by presenting themselves in large numbers in the peripheral blood during the peak biting time of the mosquito. However, the mf need not be absent from the peripheral blood during the rest of the period unless there are some physiological factors which govern their appearance and
disappearance. For example, there are several animal filariae such as the Litomosoides carinii and Dipetalonema viteae, which do not show any orientation to host circadian rhythm or vector feeding rhythm through mf periodicity (Reddy et al., 1984), and still have successful transmission.

2.3. Vectors of lymphatic filariasis

In the year 1866, Otto Wucherer discovered microfilariae of filarial parasite in the chylous urine of patients. In 1870, Timothy Richard Lewis detected filariae in the blood of a patient, and later in 1876, Bancroft in Australia could remove adult worm from an abscess on the arm of a Chinese immigrant. It was Sir Patrick Manson, in 1878, made the discovery that mosquitoes transmit the nematodes, which causes filariasis.

Mosquito species, viz., Cx. quinquefasciatus, Culex pipiens molestus, Culex pipiens pipiens, Anopheles sinensis, Anopheles gambiae, Anopheles melas, Anopheles merus, and Anopheles maculatus are natural vectors of periodic W. bancrofti. Sub-periodic W. bancrofti is transmitted by Anopheles nivensis, Anopheles oceanicus, Aedes polynesiensis, and Aedes pseudoscutellaris. In the case of B. malayi periodic form, Mansonia annulifera, Mansonia uniformis, Anopheles nigerrimus, Anopheles umbroses, Anopheles barbirostris, and An. melas are known as natural vectors. Mansonia annulata, Mansonia bonneae and Mansonia dives are the known vectors of B. malayi sub-periodic form. An. barbirostris is the vector of B. timori (WHO, 1989). Mosquitoes of the genus Culex play a vital role in the transmission of bancroftian filariasis. The Cx. pipiens complex, which includes Cx. pipiens, Cx. quinquefasciatus and Cx. molestus, belongs to the Subfamily Culicinae under the Family Culicidae, Order Diptera of Class Insecta. They act as principal vectors of the nocturnally periodic form of W. bancrofti in many parts of the tropical, sub-tropical and temperate zones including most countries of South-East Asia, coastal urbanized areas in Africa, West Indies, South America and Micronesia. Although Cx. quinquefasciatus is widely distributed in tropical and sub-tropical areas and because of the anthropophilic and endophilic blood feeding habits of the female, it
is closely associated with man and human habitations (Forattini et al., 1993; Consoli and Oliveira, 1994). It is a highly efficient mosquito host for the larval development of *W. bancrofti* (Rosen, 1955; Crans, 1973; WHO, 1980). This species was shown to be refractory to *B. malayi* in Sumatra (Lichtenstein, 1927) and in India (Iyengar, 1932). In India, the vector of *W. bancrofti* is *Cx. quinquefasciatus* (Das, 1976; Rajagopalan et al., 1977; Rajagopalan and Das, 1987). Even when different species of mosquitoes were fed artificially on heparinized microfilaraemic blood, *Cx. quinquefasciatus* showed the highest percentage of infection by *W. bancrofti* (Paily et al., 2006)

2.4. **Life cycle of lymphatic filarial parasites**

Filarial parasites require two different host systems to complete their life cycle (Plate 4). The definitive host is the man or some other vertebrate animal, depending upon the species of the parasite. The intermediate host for extrinsic life cycle is a blood sucking arthropod such as mosquitoes.

*Plate 4*: Life cycle of *Wuchereria bancrofti*

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(mf develop to L1 in the thoracic muscle of the mosquito. L3 migrate to the head of the mosquito and get transmitted to the human host through proboscis. In the human host it develop to adult and produce microfilaria)
2.4.1. In the mosquito host:

Filarial parasite development in the mosquito is not cyclo-propagative, unlike that of malaria parasites, and hence, the parasite number in the mosquito does not increase. Parasite life cycle in the vector mosquito start with ingestion of microfilariae along with the blood meal. Shortly after a blood meal is ingested, midgut cells secrete a peritrophic matrix composed of proteins embedded within chitin that separates the blood bolus from the midgut epithelium (Ponnudurai et al., 1998). It has been proposed that the peritrophic matrix provides protection against pathogens, keeps protease inhibitors within the lumen, and functions as a solid support as well as a semipermeable filter for digestive enzymes and blood proteins (Billingsley, 1990). Complete formation of the peritrophic matrix takes 12 to 30 h (Clements, 1992) and therefore, it does not serve as a formidable barrier to mf ingested by vector mosquitoes that generally enter or migrate through the midgut epithelium within a few hours. Microfilariae traverse the mosquito midgut by means of a cephalic hook that enables them to mechanically disrupt the tissues of the midgut. Exsheathment of mf takes place both in the midgut as well as in the haemocoel of the mosquito host. The mf which migrate immediately after ingestion will exsheath in the haemocoel and those remain in the midgut for more than two hours cast their sheath in the midgut itself, and then migrate (Chen and Shih, 1988). Exsheathed mf migrate anteriorly through the haemocoel and penetrate the thoracic flight muscles (Plate 5).

In thoracic muscles, they become shorter and thicker to develop into the sluggish first stage larvae (L1). At about 5th – 7th day, the L1 moult to become the second stage (L2), which is more active, and finally by 10th - 13th day they moult to become the infective stage larvae (L3). This stage is very active, showing oscillatory pattern of movement between the head, thorax and abdomen of the mosquito. On maturity, most of the L3 migrates to the head and proboscis of the mosquito to get transmitted to the mammalian host during the subsequent feeding. The L1 measures about 125 - 250 μ in length and 10 - 17 μ in width. The L2 stage larva is of 225 - 300 μ in length and 10 - 17 μ in width. The L3 is of the size of 1.2 – 1.6 mm in length and 18 – 23 μm in width (Sasa, 1976). The L3 can
remain alive and active for about 46 - 50 days, or as long as the mosquito survives (Pailly et al., 1995a).

Plate 5: Development of *Wuchereria bancrofti* in *Culex quinquefasciatus* (mf migrate to the thoracic muscles within 24 h of its ingestion. L3's migrate to the head and proboscis to get transmitted to the mammalian host)

2.4.2. In the human host:

When the mosquito feeds on the host, L3 are deposited on the skin and after withdrawal of the proboscis, they get into the wound and travel to the efferent lymphatics and sub-capsular sinus. Approximately 9 - 10 days after entry, the L3 moults to become the fourth stage larva (L4). The L4 stage goes through developmental process over several days to months, depending upon the species, to undergo the final moult and become the adult stage (L5). Adults of lymphatic filarial nematodes live in the lymphatic system of man, including the tunica vaginalis testis of hydrocoel patients (Sivam et al., 1995), and are viviparous. Ultrasound examination of male carriers of *W. bancrofti* microfilaria to detect the location of adult worms showed the typical 'filaria dance sign (FDS)' suggesting the presence of adult worms in the intrascrotal juxtesticular lymphatic vessels in 'nests' along the lymphatic vessels of the epididymis, spermatic cord and paratesticular region (Reddy et al., 2004). The adult male of *W. bancrofti* has a length of 23.8 - 30.6 mm and width of 90 - 120 μm. The length and width of adult female is respectively, 42.2 - 46.3 mm and 160 - 188 μm. The mf is of 309
- 346.8 μm in length and 5.3 μm in width (Sasa, 1976). Adult male and female mate and the gravid females release embryonic stages called mf into the lymphatic system, which migrate to the blood and get into the circulatory system of the host. It will be in higher densities in the peripheral blood on a particular time, depending upon the periodicity of the parasite. Apart from blood, mf have been demonstrated in the lymph, hydrocoele fluid, vaginal secretions, fine needle aspiration of lymph nodes and bone marrow of human carriers (Swarup et al., 1990; Sivam et al., 1995; Varghese et al., 1996; Sah et al., 2002; Sharma et al., 2006) and in the brain of B. malayi infected experimental animals (Paily et al., 1995b). The period of development and the longevity of the parasites vary according to the species of the nematode and the mammalian host (WHO, 1984). Estimation based on deterministic model showed that the life span of W. bancrofti adult female is 10.2 years. The rate of production of mf by the adult female was found to be stable at least for a period of five years (Vanamail et al., 1990).

2.5. Host-parasite interactions

The susceptibility of a mosquito species to filarial infection depends on various factors, which could be genetical, immunological, physiological or physical. Each step in the ingestion and infection process of mf in the mosquito host requires particular mechanisms and failure of any one would reduce the invasiveness. The mf entering the mosquito gut through the blood meal has to overcome a series of barriers known as the gut barriers. First of these are the cibarial and pharyngeal armatures, which in some species of mosquitoes are well-built and might damage the sheath of the mf (Coluzzi and Trabucchi, 1968; Bryan et al., 1974). Depending upon parasite size, the pharyngeal or cibarial armature, can affect infection parameters. In certain mosquito species, the pharyngeal armature can cause physical damage to large parasites, like mf, that can effectively prevent further development (McGreevy et al., 1978). Within the midgut environment, ingested parasites are exposed to proteolytic enzymes that are secreted into the lumen for blood digestion. These digestive enzymes can have a negative or positive impact on the parasites and therefore can influence vector competence (Shahabuddin et al., 1998). The process of blood feeding initiates the
formation of a chitinous structure called the peritrophic matrix within mosquitoes, which eventually surrounds the blood meal and physically separates it from the midgut epithelium. For parasites spending more than a few hours within the midgut lumen, the peritrophic matrix presents a barrier that must be traversed if they are to penetrate the midgut epithelium during migration to their developmental site. In anophelines like *An. gambiae*, *Anopheles arabiensis*, *An. melas* and *Anopheles funestus*, the mf get damaged by the mosquito foregut armatures (Bryan and Southgate, 1988). The speed of clotting of blood in the mosquito midgut varies from species to species, and the mf might get trapped within the abdomen of the mosquito if the clotting occurs before the migration of mf to the thorax (Kartman, 1953; Ewert, 1965). Also, the peritrophic membrane of certain species of mosquitoes limits the penetration of mf through the gut wall (Esslinger, 1962). The midgut wall of mosquitoes too provides a potential barrier to mf invasion and the mf has to disrupt the midgut wall to facilitate the migration. For example, following exsheathment, which takes place before or during penetration, *Brugia* spp. mf rupture the midgut epithelium by means of a cephalic hook, tearing the luminal surface and the basement lamina (Esslinger, 1962).

Limitation, facilitation and proportionality are the three types of relations observed in human filaria/mosquito couples depending upon the species of the mosquito, which ingest the mf (Southgate and Bryan, 1992). Soliman (1995) has studied the comparative exsheathment of mf of *W. bancrofti* in their vector *Cx. pipiens* and in a refractory mosquito species, *Aedes caspius*. The results showed that 94.2% of the exsheathed mf reached the thoracic region of *Cx. pipiens* and only one-tenth of this proportion was observed in the thorax of *Ae. caspius*. Failure of a large proportion of mf to exsheath after being ingested by *Ae. caspius* indicated the inefficiency of this mosquito to support filarial parasite development, contrary to its efficient migration and development in *Cx. pipiens*. Similar studies on mosquito susceptibility have concluded that the physical defense lines to invading parasites in *Cx. pipiens* are insignificant (Gad *et al.*, 1996). However, there are reports that production of polypeptide haemolymph factors in response to ingestion/inoculation of parasites and/or bacteria could limit the capacity of filariae to develop in susceptible mosquitoes (Townson and
Chaithong, 1991). The parasite load in the vector mosquito itself could act as a limiting factor for filarial parasite survival and development through its effect on the survival of the vector mosquitoes. Survival analysis of *Cx. quinquefasciatus* infected with *W. bancrofti* showed that the parasite load in the mosquito is a risk factor of vector survival (Krishnamoorthy *et al.*, 2004).

2.6. **Immune response of insects against parasites and pathogens**

As in the case of vertebrates, invertebrates also have the ability to initiate an immune response to foreign agents that infect the organism's body. However, unlike that of vertebrates, where both innate and acquired specific immunity exists, invertebrates exhibits only non-specific immune system (Hoffmann, 1995; Raymond *et al.*, 1999) though an alternative adaptive immunity in invertebrates was reported by Kurtz and Armitage (2006).

Invertebrates, which lack adaptive/acquired immune systems, have developed other systems of biological host defense, so called innate immunity, that respond to common antigens on the cell surfaces of potential parasites or pathogens. The most likely candidates for recognizing foreign material in insects are the lectins, which have already been shown to be important in mammalian innate immunity (Raymond *et al.*, 1999). During the past two decades, the molecular structures and functions of various defense components that participated in innate immune systems have been established in Arthropoda, such as, insects, the horse shoecrab, freshwater crayfish, and the protochordata ascidian (Sadaaki and Kawabata, 1998). These defense molecules include phenoloxidases, clotting factors, complement factors, lectins, protease inhibitors, anti-microbial peptides, 'Toll' receptors, and other humoral factors found mainly in the haemolymph plasma and haemocytes. These components, which together compose the innate immune system, defend invertebrate from invading bacterial, fungal and viral pathogens and parasites.

Insects are attractive model organisms for the study of innate immunity, because they lack the complexity of the vertebrate-restricted adaptive immune
responses (Hoffmann et al., 1999). The innate immune mechanisms of the insects are conserved throughout the animal kingdom. Insects have developed efficient immune responses to oppose the damaging effects of microbial and eukaryotic invaders. In response to a bacterial attack, their fat body (the equivalent of the liver in mammals) synthesizes a whole range of peptides with an antibacterial and antifungal effect. The tobacco hornworm *Manduca sexta* belonging to the Order Lepidoptera, a widely used model for insect biochemical research, employs innate immune mechanism to defend against invading pathogens and parasites (Kanost et al., 2001). *M. sexta* haemolymph showed a group of proteins like haemolin, peptidoglycan recognition proteins, β-1,3-glucan recognition proteins, and C-type lectins. These groups of proteins serve as surveillance mechanisms by binding to microbial surface molecules like peptidoglycan, lipopolysaccharide, lipoteichoic acid, and β-1,3-glucan. The binding triggers diverse responses such as phagocytosis, nodule formation, encapsulation, melanization, and synthesis of anti-microbial peptides/proteins. The immune protein, scolexin, a bacteria-induced, larva-specific protein from *M. sexta*, was shown to exist in the haemolymph in two isoelectric forms designated as scolexin-1 and scolexin-2 (Kyriakides et al., 1995). Up-regulation of protease inhibitors that regulates phenoloxidases called serpins (serine-protease inhibitors) were described in bacteria challenged *M. sexta* haemolymph (Tong and Kanost, 2005; Zhu et al., 2003). Immulectin-2, a C-type lectin, which enhances encapsulation and melanization processes was reported in, *M. sexta* (Yu and Kanost, 2003). Other immune genes with anti-microbial activities like cecropin, haemolin and lysozyme were also described in this insect (Dickinson et al., 1988; Ladendorff and Kanost, 1991; Wang et al., 1995; Mulnix and Dunn, 1994). Recently, analysis of the host - polydnavirus interactions with the Lepidopteran pest, *Spodoptera frugiperda*, by a microarray approach indicated variations in transcript levels of specific host immune genes of calreticulin and melanization activating enzymes (Barat et al., 2006).

Haemolin is a bacteria-inducible protein of the immunoglobulin superfamily identified in the silk moth *Hyalophora cecropia*. The role of this protein, in haemocyte aggregation and phagocytosis, was studied *in vitro* (Lanz-Mendoza et al., 1996) and its up-regulation was also evidenced (Roxström-
The other immune genes described in this insect includes attacin A and B (Kockum et al., 1984; Lee et al., 1983), cecropin A, B and D (Lidholm et al., 1987), and lysozyme (Engstrom et al., 1985; Sun et al., 1991). Similarly, two cecropin A genes were cloned and sequenced from the silkworm, *Bombyx mori* (Yamano et al., 1998). The other immune genes described in *B. mori* includes, attacin (Sugiyama et al., 1995), cecropin (Kato et al., 1993; Taniai et al., 1995), lebocin (Chowdhury et al., 1995), and lysozyme (Lee and Brey., 1994). A novel insect defensin with potent antifungal activity was isolated from *Heliothis virescens* (Lamberty et al., 1999). Viresin is a novel antibacterial protein from immune haemolymph of *H. virescens* (Chung and Ours, 2000).

Mammalian apolipoproteins such as apolipoprotein E (apoE) are involved in LPS detoxification, phagocytosis, and possibly pattern recognition. The multifunctional insect protein, apolipoporphin III (apoLp-III), homologous to apoE and involved in pattern recognition and multicellular encapsulation reactions was isolated from the wax moth, *Galleria mellonella* (Whitten et al., 2004). Inhibition of protein kinase A (PKA) which alters the humoral immune response was also described in the same moth (Cytrynska et al., 2006; Cytrynska et al., 2007).

Insect defensins are mainly active against Gram-positive bacteria and appear to have a large distribution within the Class of the insects. Their presence have been reported in the Orders Diptera, Coleoptera, Hymenoptera, Trichoptera, Hemiptera and Odonata (Lambert et al., 1989; Hoffmann and Hetru, 1992; Lowenberger et al., 1995; Bulet et al., 1991; Cociancich et al., 1993). A 43-residue cysteine-rich anti- Gram-positive bacteria peptide belonging to the family of insect defensins was isolated from the sap-sucking bug *Pyrrhocoris apterus*, a representative species of the Hemiptera (Cociancich et al., 1994). Among Coleopteran insects, presence of defensin molecule with activity against Gram-positive bacteria was demonstrated in the larvae of *Anomala cuprea* (Yamauchi, 2001). A new serine protease regulating prophenoloxidase activation during immune reaction was identified in the larvae of *Holotrichia diomphalia* (Kim et al., 2002). Similarly, a novel 43-kDa protein, as a negative regulatory component of phenoloxidase-induced melanin synthesis was described from the meal-worm *Tenebrio molitor* (Zhao et al., 2005). The humoral immune response of the
American cockroach, *Periplaneta americana* was described by Rheins and Karp (1985), in which the female primary response was found to be enhanced and prolonged as compared to the male. Also, the response was specific with long-term immunological memory. Thus, the humoral immune response of the American cockroach shares a characteristic feature common to higher vertebrates, i.e., the female of the species demonstrated much better immune responsiveness than the male. In yet another interesting study, Whitten *et al.* (2001) described the role of NADPH oxidase and nitric oxide synthase in the immune response of the vector bug *Rhodnius prolixus*, to infection by *Trypanosoma rangeli*, the causative organism of chagas disease. Very recently, antiserum raised against perimicrovillar membranes and midgut tissue of *R. prolixus* was found to interfere with the midgut structural organization and reduced the development of *Trypanosoma cruzi* (Gonzalez *et al.*, 2006). An ant-imicrobial peptide belonging to the defensin family was also isolated from the haemolymph of *R. prolixus* (Lopez *et al.*, 2003).

2.6.1. **Immune responses in Dipteran insects:**

Genetic and molecular studies on humoral defenses of *Drosophila melanogaster* have greatly contributed to our understanding of innate immunity in Dipteran insects (Hoffmann, 2003). However, cellular immune responses such as phagocytosis (Elrod-Erickson *et al.*, 2000; Lemaitre and Hoffmann, 2007), which have been studied in this model insect remains less understood. In the last four years, two new recognition molecules that contribute to microorganism binding and/or engulfment have been characterized using S2 *Drosophila* cells (Ramet *et al.*, 2002). They are scavenger receptor I (dSR-CI), which facilitates binding of Gram-negative and Gram-positive bacteria to these cells, and PGRP-LC, a peptidoglycan recognition protein that is involved in the phagocytosis of Gram-negative, but not Gram-positive, bacteria.

Hoffmann (1995), described mechanisms that control anti-microbial response in insects, using *Drosophila* as the model. Availability of the *Drosophila* genome has enabled several research groups to use proteomic and genomic
approaches to study the complex processes involved in the innate immune defense of this insect against microorganisms (Engstrom et al., 2004).

Several different control pathways governing the expression of the genes that code for anti-microbial peptides have been identified in *Drosophila*. One of these cascades includes the ‘Toll’ pathway, which is structurally and functionally similar to the specific pathway in mammals responsible for the expression of the genes involved in the acute phase immune response (Bian et al., 2005). It indicated that the cascade involved in the immune response must have appeared early in the evolution of eukaryotes. It also illustrates the striking similarities between the anti-microbial response in insects and the innate, nonadaptive response in mammals (Hultmark, 1993). However, the ability to use genetic recombination to produce proteins with diverse antigen binding sites, such as those of immunoglobulins and T-cell receptors, appears to have evolved after the divergence of vertebrates and invertebrates (Faye and Kanost, 1998). The NF-kappaB group of transcription factors plays an important role in mediating immune responses in organisms as diverse as insects and mammals. Microarray analysis of the fruit fly *D. melanogaster* have been shown to express three closely related NF-kappaB- like transcription factors such as Dorsal, Dif, and Relish (Pal et al., 2007). In insects, transcription of immune genes are induced during bacterial infection and results in the secretion of antibacterial peptides into the haemolymph. This immune response is very fast as it takes only hours before the peptides appear in the blood, but is non-specific as the same set of peptides is induced upon infection with different bacteria (Casteels, 1997). The main advantage of the antibacterial peptides as factors of innate immunity is that they can function without high specificity or memory.

In insects, antimicrobial peptides (AMPs) are promptly synthesized at low metabolic cost, easily stored in large amounts, and readily available shortly after an infection. AMPs are also locally produced by barrier epithelia of several insect species such as, *An. gambiae* (Brey et al., 1993; Dimopoulos et al., 2002; Richman et al., 1996), and *D. melanogaster* (Ferrandon et al., 1998; Tingvall et al., 2001). In *D. melanogaster*, other than anti-microbial peptides, C-type lectins, which are calcium-dependent carbohydrate binding proteins, also participate in
innate immunity (Beck and Strand, 2005). In this Dipteran species, more than 30 genes encoding C-type lectin domains were described recently (Ao et al., 2007). The inducible expression of the anti-microbial peptide genes in D. melanogaster is regulated by the conserved ‘Toll’ and peptidoglycan recognition protein LC/immune deficiency (PGRP-LC/IMD) signalling pathways. It has been proposed that the two pathways have independent functions and mediate the specificity of innate immune responses towards different microorganisms (Cherry and Silverman, 2006; Tanji et al., 2007). The role of one of these two major pathways regulating innate immunity, the immunodeficiency (Imd) pathway, was well characterized in Glossina morsitans’s natural defense against T. brucei infections. Using RNA interference, it was shown to have highest homology with D. melanogaster Imd pathway (Hu and Aksoy, 2006). Analysis of the haemolymph of bacteria and T. brucei- challenged adults of G. morsitans by reverse-phase chromatography and mass spectrometry have identified three AMPs such as a cecropin, an attacin, and a defensin (Boulanger et al., 2002). To date, several AMPs have been described in immune- challenged insects, where they exhibit wide and complementary spectra of activities against various microorganisms (Bulet et al., 1999).

2.6.2. Immune response of mosquitoes:

The efficiency of vector-borne disease transmission depends on the interplay between the vector and the parasite/pathogen. The haematophagous female mosquito requires blood from vertebrate hosts to complete its gonotrophic cycle. The biochemical make up of this ingested blood can have a significant influence on vector competence. There are three possible outcomes following exposure of a vector mosquito to a parasite/pathogen via an infective blood meal, such as: (i) in a susceptible mosquito, the parasite/pathogen receives the appropriate stimuli from a compatible biochemical environment and develops and/or reproduces successfully, (ii) in a resistant mosquito, some or all of the parasites/pathogens are recognized as foreign by the cellular/humoral components of the immune system, and it initiates an immune response that can effectively sequester and destroy the parasites/pathogens, or (iii) in a refractory mosquito, the parasites/pathogens do not elicit an immune response and although they can be
successful in their migration to and invasion of the appropriate tissue, they fail to develop due to a physiological or biochemical incompatibility.

Thus, vector competence, as a component of vectorial capacity, is governed by intrinsic or genetic factors that influence the ability of a vector to transmit a pathogen (Black and Severson, 2005). Any trait, for example, host feeding preferences or susceptibility to parasite/pathogen infection, that has a genetic component will affect vector competence. Following ingestion by mosquitoes through a blood meal, parasites/pathogens encounter various physical barriers that regulate its survival. This will be less or will not be sufficient at all in vector mosquitoes. Parasite/pathogen developmental sites, which includes the midgut, malpighian tubules, thoracic muscles, and salivary glands, can provide the appropriate environment to initiate parasite gene expression or permit post-translational modifications of previously expressed gene products that are required for development. Consequently, the composition of these tissues within specific species and strains of mosquitoes plays a major role in the vector competence. In many incompetent vectors, parasites successfully reach the appropriate developmental site, but then fail to develop or are killed by a defense response (Christensen and Tracy, 1989). The salivary glands of mosquitoes synthesize and secrete powerful anti-haemostatic agents that facilitate haematophagy by counteracting the effects of vertebrate wounding responses (Ribeiro, 2000). In *Ae. aegypti*, these anti-haemostatic molecules include apyrases, sialokinins, and anticoagulants that prevent, respectively, the platelet aggregation, vasoconstriction, and coagulation (Jahan and Hurd, 1997).

The mosquito midgut is a potentially hostile environment for parasites or pathogens, because the blood within the midgut is different from the blood circulating in a homeothermic organism. Exiting the midgut as quickly as possible would seem to be a viable strategy for most mosquito-borne parasites or pathogens. Filarial worms and viruses do exit quite rapidly, but malaria parasites, which may spend over 24 h in the midgut, take advantage of this environment as the stimulus for sexual reproduction (Berner *et al.*, 1983; Garcia *et al.*, 1998). As a result, *Plasmodium* spp. face different obstacles, such as extended exposure to proteolytic enzymes, from those faced by filarial worms or viruses. Analysis of
anti-microbial gene expression within mosquito midguts has demonstrated differences in immune system activation between _An. gambiae_ and _Ae. aegypti_. Up-regulation of mRNA transcripts for anti-microbial genes, such as defensin, have been observed in the midguts of _An. gambiae_ exposed to either bacteria or a _P. berghesi_ infected blood meal (Rossignol _et al._, 1984, Dimopolous _et al._, 1997) However, in the midgut of _Ae. aegypti_, no such up-regulation was seen (Lowenberger _et al._, 1996)

At some time during their life cycle within the mosquito, almost all parasites/pathogens must travel through the hemolymph-filled haemocoel, when migrating to their developmental site or to their site for transmission to the vertebrate host. Haemolymph is the functional tissue responsible for many of the immune responses in insect and it consists of haemocytes and plasma. The primary immune components involved in the recognition of non-self and in the initiation of defense responses designed to kill foreign invaders reside within the haemolymph (Christensen and Tracy, 1989). The cellular portion of the haemolymph is composed of circulating haemocytes which includes granulocytes, plasmatocytes, oenocytes, etc. Vector immune responses elicited by invading organisms is robust and are often separated into cellular and cell-free, or humoral responses. The cellular component includes phagocytosis (Hernandez _et al._, 2002, Hillyer _et al._, 2005), and encapsulation (Forton _et al._ 1985, Christensen and Forton, 1986) by haemocytes. The humoral component includes the phenoloxidase cascade system of parasite melanization and wound healing (Zhao _et al._ 1995, Lai _et al._, 2002), inducible anti-microbial peptides (Lowenberger, 2001, Vizioli _et al._, 2001), and reactive oxygen and nitrogen intermediates (Luckhart _et al._ 1998, Lanz-Mendoza _et al._, 2002, Kumar _et al._ 2003)

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Haemocytes have multifaceted cell functions as pattern recognition, phagocytosis, melanization, and signalling cascades that initiate varying cytotoxic effector responses. They are the central element mediating systemic mosquito innate immune responses and are the major phagocytic cells in mosquitoes (Barreau _et al._, 1995, Hillyer _et al._, 2003). Within seconds after injection into the
mosquito thorax, bacteria are distributed in the whole mosquito body by fast flow of the haemolymph. Sessile haemocytes come in contact with the bacteria and fix it on the surface, probably with the help of surface recognition molecules. It takes only minutes for haemocytes to engulf bacteria (Hillyer et al., 2003). Interestingly, mosquito haemocytes phagocytose Gram-negative bacteria more readily than Gram-positive (Hernandez et al., 2002; Hillyer et al., 2004). Another essential function of haemocytes is the synthesis of components of basal membranes. Some of these molecules, such as laminin, b-integrin1, are detected on the surface of parasites, suggesting that the components of basal lamina are interacting with parasite surface proteins (Dessens et al., 2003; Mahairaki et al., 2005). Another molecule produced by haemocytes, TEP1, belongs to the family of thioester-containing proteins (TEPs), and share significant similarities with the vertebrate complement factors C3, C4 and C5, and with a2-macroglobulins (Bartholomay et al., 2004).

Phagocytosis is a classical cellular immune response of insects whereby haemocytes engulf the target pathogens as well as any other apoptotic bodies. It is a primary defense mechanism against bacteria and other particles, such as various protozoan spores, which are small enough to be engulfed. Evidence for phagocytic events is manifested in large number of signal transductions, cytoskeletal rearrangements, and apoptotic elements (Bullet et al., 1993). It has been described in the haemolymph of many insect species against biological (Ratcliffe and Rowley, 1979; Ratcliffe et al., 1985; Götz and Boman, 1985) as well as non-biological agents (Wiesner, 1991; Slovak et al., 1991). The malaria vector An. gambiae and many other species of mosquitoes mount powerful immune responses, including phagocytosis, against a wide range of microorganisms (Levashina et al., 2001; Hillyer et al., 2004). Phagocytic cells in Ae. aegypti and Armigeres subalbatus, have the capacity to engulf hundreds of bacteria, which undoubtedly requires extensive remodelling of cytoskeletal elements, including actin, actin-binding and polymerizing factors, and alpha- and beta-tubulins. If the parasite/pathogen is too large to be phagocytosed, such as filarial worm, mosquito haemocytes may recognize the parasite/pathogen as foreign and recruit other haemocytes to participate in the melanization response, rather than phagocytosis.
The mechanisms promoting cellular encapsulation in insects are not well understood, and only a few examples of this reaction have been reported. Encapsulation is a process by which insect lamellocytes form a multilayered capsule around large invaders such as parasitoids in the haemocoel, resulting in their isolation, immobilization and subsequent killing by asphyxiation, oxidation or melanization (Gotz, 1985). Mosquito larvae encapsulate and then melanize the eggs of a parasitoid wasp in their haemocoel through the concerted action of lamellocytes and crystal cells (Lanot et al., 2001; Sorrentino et al., 2002).

Melanization is a prime humoral immune reaction of insects, being involved in wound healing and sequestration of invaders in a dense melanin coat. In contrast to encapsulation, it does not require the direct involvement of haemocytes (Soderhall and Cerenius, 1998). Activated haemocytes produce transferrin, which is a melanization-associated molecule, and a prophenoloxidase (ProPO), which is activated by a serine protease to become phenoloxidase (PO). After a phenoloxidase-catalyzed hydroxylation of tyrosine, other enzymes like dopa-decarboxylase (DDC) and dopachrome conversion enzyme (DCE) catalyze other critical reactions. Ultimately, it results in melanin production around the parasite/pathogen. Melanization, thus involves a complex series of reactions requiring tyrosine precursors and phenol oxidases to produce a capsule, consisting of melanin polymers cross-linked with proteins, that sequesters the foreign invader (Christensen and Tracy, 1989). This response is related to the normal cuticular sclerotization, egg chorion tanning, and wound-healing pathways, and a number of substrates and enzymes are shared among these diverse biochemical events (Brunet, 1980; Czapla et al., 1989). Among the dopa- and dopamine oxidation pathways, most likely the dopamine pathway is involved in melanization, because the intermediates are longer-lived and more stable, thereby making them more readily available for cross-linking with proteins involved in melanotic capsule formation (Li, 1994). This response has been observed in all mosquitoes studied to date, including the species of mosquitoes susceptible to parasite development (Paskewitz and Christensen, 1997). It is a specific immune response and the extent and effectiveness of the response may be influenced by the parasite/pathogen (Christensen et al., 1986; Collins et al., 1986). For example, natural and laboratory populations of *Aedes subalbatus* melanotically encapsulate...
B. malayi microfilariae but are unable to melanize the microfilariae of closely related B. pahangi. Similarly, in the vector mosquito Cx. quinquefasciatus, the mf of W. bancrofti develop to infective larvae without the known host responses such as melanization or encapsulation. However, as in the case of blackfly, whether there is any humoral response against the invading parasite, which regulate the infection, further development and subsequent transmission of the disease is not known. If it exists, the means by which the mosquito defends itself and limit the parasite development are important factors determining its vector competence. Repeated feeding of Cx. quinquefasciatus with W. bancrofti mf positive blood was found to reduce the parasite burden in the subsequent infection (Paily et al., 1995c) and that could be due to the immune response of the mosquito challenged with W. bancrofti.

2.6.2.2. Humoral immunity:

The synthesis of AMPs is the final step of inducible humoral immune responses, which includes the production of immune peptide molecules, whereby mosquitoes protect themselves against pathogens. Some of the known AMPs produced by mosquitoes, especially Anopheles and Aedes mosquitoes, are cecropin, defensin and gambicin. The mosquito immune peptides have been classified according to their physical structure and include cysteine-rich defensins, proline-rich and glycine-rich compounds, and α-helix-containing cecropins (Collins et al., 1996; Hetru et al., 1998; Bulet et al., 1999). In mosquitoes, defensins and, to a lesser extent, cecropins have been well described, but little information is currently available concerning proline- and glycine-rich compounds.

Following pathogen recognition, AMPs are rapidly produced by the fat body as well as the haemocytes and secreted into the haemolymph, where they accumulate in large concentrations (Meister et al., 1997). In Drosophila, the AMP-encoding genes are regulated by finely tuned activity of IMD and ‘Toll’ pathways (Bulet et al., 1999; Hoffmann, 2003; Hoffmann and Reichhart, 2002). IMD controls the expression of anti-microbial peptides such as diptericins and drosocins, while ‘Toll’ induces the expression of the antifungal peptide
drosomycin. Signals from both ‘Toll’ and IMD seem to control jointly the induction of cecropins, attacins and defensins (Hoffmann et al., 1996; Meister et al., 1997). Comparative transcriptomic analysis of Drosophila with An. gambiae revealed a strong similarity of developmental expression between orthologous genes (Koutsos et al., 2007). The genome sequence of An. gambiae harbours only two NF-κB genes, REL1 (Gambfl) and REL2 (Relish), which are homologues of Drosophila Dorsal and Relish (Bian et al., 2005), respectively. It has also been shown that Ae. aegypti REL1 regulates antifungal immune response and functions as a Dif analog of the ‘Toll’- mediated antifungal immune pathway of Drosophila.

Cecropin, isolated from the Lepidopteran insect H. cecropia was the first characterized antibacterial immune peptide (Steiner et al., 1981). Matured cecropin peptides contain 35 - 39 amino acids, excluding cysteines, and form two α-helices separated by a hinge. It is a small peptide of 4- kDa with broad-spectrum activity against Gram-positive and Gram-negative bacteria. It has been isolated from a bacteria-challenged Ae. albopictus cell line and the resulting peptide sequence was found to exhibit 36% identity to cecropin from D. melanogaster (Hernandez et al., 2003). Although produced in response to bacterial infection, immune peptides have been assayed for their ability to prevent or limit infection of mosquitoes by eukaryotic organisms such as filarial worms and malarial parasites. Gwadz et al. (1989) demonstrated that when cecropins, derived from the skin of frogs or giant silk moths were injected into Anopheles infected with Plasmodium spp., normal oocyst development was disrupted. In another study, co-injection of B. pahangi microfilariae with cecropins into Ae. aegypti significantly reduced the numbers of developing filarial larvae, compared with those in controls (Chalk et al., 1995).

Defensins are the first family of anti-microbial peptides identified in mosquitoes (Chalk et al., 1994; Lowenberger et al., 1995; Richman et al., 1996). In Ae. aegypti and An. gambiae, defensins are produced and secreted by the fat body and midgut tissues as precursor molecules. These are small cationic peptides characterized by six cysteine residues engaged in three intramolecular disulfide bridges. Defensins have a highly conserved 3D structure and consists of an N-terminal loop, and an α-helix and two antiparallel β-sheets (Cornet et al., 1995).
Two closely related defensins A/B and C were identified and characterized in *Ae. aegypti* (Lowenberger, 2001). However, in *An. gambiae*, the defensin family includes four divergent molecules (Christophides *et al*., 2002).

The activity spectrum of mosquito defensins is directed primarily against Gram-positive bacteria, although some Gram-negative bacteria are also sensitive to them (Lowenberger *et al*., 1995; Vizioli *et al*., 2001). They are the predominant immune peptides produced in bacteria challenged *Ae. aegypti* (Chalk *et al*., 1995; Lowenberger *et al*., 1995). In this mosquito, the defensin levels were shown to be strongly up-regulated following bacterial challenge, reaching a concentration of approximately 45 μmol l⁻¹ in the haemolymph at 24 h post-inoculation (Lowenberger *et al*., 1999). Recently, defensin was identified in *Cx. pipiens* after infection with the filarial parasite, *W. bancrofti* (Bartholomay *et al*., 2003). Injection of purified defensins from the dragonfly, *Aeschna cyanae*, and the flesh fly, *Phormia terranovae*, into *Ae. Aegypti* has been reported to reduce the establishment of *P. gallinaceum* (Shahabuddin *et al*., 1998). Additionally, purified endogenous defensin reportedly has a negative influence on the development of *B. pahangi* when injected into *Ae. aegypti* (Albuquerque *et al*., 1996). *In vitro* studies indicated that defensins had no detectable effects on the early stages of malarial parasites, such as zygotes and ookinetes, but were toxic to the sporozoite stage (Tahar *et al*., 2002). Bacterial challenge of mosquitoes followed by an infectious blood meal containing either the filarial worm *B. malayi* or the malaria parasite *P. gallinaceum* or *P. berghei* were found to result in a significantly reduced prevalence and mean intensity of infection, compared to that in controls with no immune system activation, and was suggested that defensins played a significant role in the observed parasite reductions (Lowenberger *et al*., 1996; Lowenberger *et al*., 1999). Very recently, phylogenetic analysis of defensin sequences revealed two main lineages, one group comprising mainly Lepidopteran insects and a second, comprising Hemipteran, Coleopteran, Dipteran and Hymenopteran insects (Dassanayake *et al*., 2007).

The anti-microbial peptides, gambicins, were identified so far only from mosquitoes. The first peptide was isolated from *An. gambiae* cell line by biochemical methods and through differential display PCR. The 616-bp
Gambicin gene encodes a 81-residue precursor protein that is processed and secreted as 61-aa mature peptide containing eight cysteines forming four disulfide bridges (Vizioli et al., 2001). Under in vitro conditions, matured peptide displayed anti-bacterial activity, as well as a morphogenic effect on a filamentous fungus. A homologous sequence was identified from the salivary gland transcriptome of *Ae. aegypti* and an EST library from *Cx. pipiens* (Bartholomay et al., 2003).

Transferrin is another protein up-regulated in mosquitoes after immune system activation. An 84-kDa protein, later identified as transferrin, was detected in haemocytes and preferentially expressed in the haemolymph of *Ae. aegypti* that were melanotically encapsulating *D. immitis* mf (Beernsten and Christensen, 1990). It has also been reported that transferrin is up-regulated in the *Ar. subalbatus - B. malayi* model system, where ingested mf are melanized rapidly. Blood feeding alone did not significantly up-regulate transferrin transcription, and even in the absence of a blood meal, injection of bacteria could increase transferrin mRNA levels (Beernsten et al., 2000). Apart from this, transferrin was reported to be up-regulated in *Ae. aegypti* and *Ae. albopictus* cell cultures, following treatment with heat-killed bacteria (Yoshiga et al., 1997). These reports suggest that at least in some mosquito species studied, transferrin is involved both in antibacterial responses and during melanization reactions.

Molecular characterization of mosquito transferrin has shown similarity with transferrin from other insect species, and it has conserved iron-binding residues in the N-terminal lobe. Unlike vertebrate transferrins, it lacks iron-binding residues in the C-terminal lobe (Yoshiga et al., 1997). It has been hypothesized that the N-terminal lobe allows transferrin to sequester iron from invading organisms, and the absence of the C-terminal lobe may be a mechanism to prevent the act of "iron piracy" (Cornelissen and Sparling, 1994), whereby bacteria preferentially pirate iron from receptors located on the C terminus of the transferrin molecule (Yoshiga et al., 1997).

Up-regulation of immune-responsive serpin, SRPN6, was described in *An. stephensi* and *An. gambiae* after infection with the malarial parasite, *P. berghei*
(Abraham et al., 2005). In insects, haemolymph coagulation, AMP production, and melanotic encapsulation, are modulated by various serine proteases and serpins i.e., serine protease inhibitors. Apart from these, thioester-containing proteins (TEPs) were also reported to play roles in the innate immune responses of Dipteran insects. TEPs are represented by multi-member families, both in the fruitfly *D. melanogaster*, and in the mosquito, *An. gambiae*. TEP1 is moderately up-regulated by bacterial and parasite infections. Phylogenetic analysis of the family suggested that in these two Dipteran species, TEPs followed independent evolution as a result of specific adaptation to distinct ecological environments (Blandin and Levashina, 2004). Double-stranded RNA knockdown experiments in adult mosquitoes indicated that TEP1 plays an essential role in the mosquito antiparasitic response (Blandin et al., 2007). In susceptible mosquitoes, the knock down of TEP1 resulted in a five-fold increase in the number of oocysts developing on the midgut, suggesting that parasite killing in mosquitoes is mediated by TEP1. Indeed, in the refractory mosquitoes, ookinetes are melanized after they complete their passage through the mosquito midgut. Knockout of TEP1 in the refractory strain resulted in complete abolishment of parasite melanization and normal parasite survival. Approximately 80% of the ookinetes were killed and subsequently lyzed in the susceptible mosquitoes, suggesting that melanization is not a mechanism of parasite killing but rather a mechanism of clearance of dead parasites in the refractory strain, similar to clearing of dead parasites by lysis in the susceptible mosquitoes (Blandin et al., 2002). Elucidation of the molecular mechanisms of TEP1 parasite killing will provide important insights into the evolution and function of the TEP family.

Very recently, expression of two fat body genes involved in lipid transport metabolism, such as lipophorin (*Lp*) and its lipophorin receptor (*LpRfh*), was reported to be significantly increased after infection of *Ae. aegypti* with Gram-positive bacteria and fungi, but not with Gram-negative bacteria. Expression of these genes was described to be enhanced after infection with *P. gallinaceum*. RNA interference (RNAi) knockdown of *Lp* strongly restricted the development of *Plasmodium* oocysts, reducing their number by 90% (Cheon et al., 2006). Peptides such as cysteine-rich drosomycin, proline-rich drosocins and metchnikowin, glycine-rich attacins, and a proline- and glycine-rich diptericin
present in *Drosophila* (Bulet *et al.*, 1999) are not reported from mosquitoes. As the drosomycin and diptericin genes are often used to follow activation of the two signalling pathways that control expression of anti-microbial genes in *Drosophila*, it is difficult to draw direct parallels between signalling cascades in the fruit fly and the mosquito (Levashina, 2004).

The components of the ‘Toll’ and Imd pathways are present in *An. gambiae* and *Ae. aegypti*, but little experimental evidence is available about the regulation of expression of anti-microbial peptide genes in mosquitoes. The pioneering studies in *Ae. aegypti* using transgenic mosquitoes expressing a dominant-negative form of the Rel-like transcriptional activator Relish pointed to the importance of Relish in regulation of expression of the defensin A/B and cecropin genes (Shin *et al.*, 2003). Furthermore, in these studies, transgenic mosquitoes were susceptible to infections with Gram-negative bacteria. The susceptibility was rescued by over-expression of the defensin A/B gene under the control of the vitellogenin promoter. These results demonstrated that mosquitoes use Rel like transcriptional factors to control the expression of anti-microbial peptide genes as in the case of *Drosophila* (Dimopoulos *et al.*, 2001).

Mosquitoes can be challenged by infection with a parasite either through injection or through artificial feeding of an infected blood meal, and the influence of the various immune peptides on parasite development can be evaluated by measuring the prevalence and mean intensity of infection at appropriate times following exposure. The effects of specific immune peptides on parasites can be studied by injecting appropriate peptides into parasite- infected mosquitoes. Injection of cecropins into mosquitoes has been reported to cause a significant reduction in *Plasmodium* development (Gwadz *et al.*, 1989; Jaynes *et al.*, 1989; Rodriguez *et al.*, 1995; Possani *et al.*, 1998; Boisbouvier *et al.*, 1998). Similarly, co-injection of cecropins or defensins with *B. pahangi* into mosquitoes was reported to reduce parasite establishment (Chalk *et al.*, 1995; Albuquerque and Ham, 1996). Injection of defensin at specific time points after blood feeding was reported to reduce the mean intensity of infection, but not the prevalence, of *P. gallinaceum* in *Ae. aegypti* (Shahabuddin *et al.*, 1998). It has been reported that immune activation of mosquitoes, via bacterial inoculation 24 h prior to parasite
exposure, reduce both the prevalence and mean intensity of infection with *B. malayi* and *P. gallinaceum* in *Ae. aegypti* and *P. berghei* in *An. gambiae* (Lowenberger *et al.*, 1996; Lowenberger *et al.*, 1999). In the case of filarial worms, the prevalence was reduced from 92–97% to 50–57%, and the mean parasite burden from 8–16 to 2.5 worms/infected mosquito. Investigations into anti-microbial gene expression have been limited mostly to *Anopheles* and *Aedes*. Relatively little information concerning immune system peptide activation or synthesis exists for *Culex* and *Mansonia* mosquitoes, despite their importance as human disease vectors.

2.6.3. **Immune evasion by parasites:**

Many parasites are able to evade or suppress the immune response of the mosquito host and successfully develop within the host. However, the mechanisms by which the parasites avoid the immune response are relatively unknown. Various phases in which the parasite can evade hosts immune response includes level of recognition, signal transduction, or production of immune system effector molecules. It has been proposed that parasites may avoid recognition as non-self by acquiring host antigens or by expressing, on their surface, molecules that exhibit identity to host antigens (Damian, 1997). In some cases, parasites may also produce secretory/excretory products that suppress the immune response of the mosquito (Christensen and Lafond, 1986). It has been demonstrated that *Ar. subalbatus* recognized and melanized *B. malayi* mf (Yamamoto *et al.*, 1985; Beernsten *et al.*, 1989), but failed to melanize mf of the closely related *B. pahangi*. In another study, melanization of inoculated *B. pahangi* was significantly decreased in *Ae. aegypti* harbouring a developing *B. pahangi* infection, compared with that in non-infected controls (Christensen and Lafond, 1986). Similarly, *An. gambiae* and *Armigeres albimanus* could melanotically encapsulate many different strains of *P. falciparum* but were unable to melanize sympatric strains of the parasite (Collins *et al.*, 1986). These reports suggest that parasites that develop successfully in the mosquito may lack immune system-stimulatory surface molecules or share host antigens, or produce secretory/excretory products that inhibit the immune response by interfering with either the recognition process, or signalling pathways, or effector mechanisms.
2.7. Importance of information on mosquito immune system

The mechanisms used by the mosquito immune system to regulate parasite development, as well as the strategies used by parasites to avoid recognition and suppression, are perhaps the most important determinants of vector competence. "Understanding the genetics behind pathogen-immune system interactions in disease vector mosquitoes may help to understand why, for example, some types of mosquitoes can transmit a particular human pathogen while others cannot" (Fatos, 2007).

As early as in 1929 and 1931, Huff demonstrated that the susceptibility of *Cx. pipiens* to an avian malaria parasite could be increased through selective mating strategies. Subsequent experiments with filarial worms and malarial parasites proved the genetic basis for susceptibility of several mosquito species to these parasites (Christensen and Severson, 1993). Availability of a genetic linkage map for *Ae. aegypti*, based on isozyme and morphological mutant markers, enabled a number of researchers to determine the chromosomal regions of genes with a major influence on the susceptibility of this mosquito species to *P. gallinaceum* (Kilama and Craig, 1969; Munsterrm and Craig 1979), *Brugia* spp. (Macdonald, 1962; Macdonald and Ramachandran, 1965; Marinotti et al., 1996), and *D. immitis* (Mcgreevy et al., 1978). Recent advances in genomics, made it possible to develop physical and linkage maps for different mosquito species, primarily *Ae. aegypti* (Severson et al., 1993), and *An. gambiae* (Zheng et al., 1996). Though *Ae. aegypti* is a natural vector of *P. gallinaceum*, and a group of flaviviruses that cause dengue fever and yellow fever, mutants that support complete development of *B. malayi, B. pahangi* and *D. immitis* also have been selected (Macdonald, 1965; Macdonald and Ramachandran, 1965; McGreevy et al., 1978). Most of the data on mosquito physiology, genetics, and vector competence have been derived from studies on *Ae. aegypti*. Successful application of double-stranded RNA interference (dsRNA) to silence *An. gambiae* gene expression *in vivo* (Blandin et al., 2002) and the sequencing of the genome of this mosquito (Holt et al., 2002) have opened unprecedented opportunities to dissect immune responses at the molecular level in living mosquitoes.
With the *Ae. aegypti* and *An. gambiae* experimental models, requisite information exists now to isolate the genes controlling susceptibility of these vectors to several different parasites/pathogens. After a blood meal, strains of *Ae. aegypti*, refractory to *B. malayi* produce seven peptides that are not found in a susceptible strain (Wattam and Christensen, 1992). Discovery of specific peptides in filaria-resistant strain of blood-fed *Ae. aegypti* opens the possibility of using molecular approaches to study the mode of action of these peptides. Furthermore, these peptides may provide a lead to transgenic approaches for filaria control in mosquitoes such as *Cx. quinquefasciatus*, the major vector of human lymphatic filariasis, in which selection for refractoriness has so far been unsuccessful (Singh and Curtis, 1974; Zielke and Kuhlow, 1977). Perhaps, the gene knockout techniques (Olson et al., 1996) and the development of an efficient and stable germ-line transformation system (James, 1994) may provide reliable methods for testing the influence of vector competence of candidate genes contained within the DNA. However, the use of molecular marker technologies to define genes controlling vector competence have, to date, involved only model systems such as *Ae. aegypti* and *An. gambiae*. This may not necessarily reflect a natural vector-parasite interaction as *Ae. aegypti* is not a natural vector of *B. malayi*, and *P. gallinaceum* and *P. cynomolgi* are not human pathogens. Undoubtedly, the isolation and characterization of a gene that imparts susceptibility or refractoriness of a mosquito to a parasite would be a tremendous accomplishment, and would provide a significant improvement of our understanding of the mechanisms controlling vector competence.