Developing transgenic models to induce late-life mortality in the malaria vector *Anopheles gambiae*

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Declaration of Originality

I declare that the intellectual content of this thesis is the product of my own research work. Any ideas or quotations from the work of other people are fully acknowledged in accordance with the standard referencing practices.

Abstract

A key factor that limits the transmission of malaria is the age of its vector, *Anopheles gambiae*. This is due to the long extrinsic incubation period (EIP) of the parasite which can last a high proportion of the adult mosquito's life. Therefore, only a small percentage of a mosquito population is able to transmit malaria. This bottleneck could be of use in developing novel vector control strategies which are based on artificially shortening the adult life span of mosquitoes through the introduction of suitably tailored transgenic constructs.

One strategy was to induce a toxic late-acting amino acid metabolism disease in mosquitoes that kills females after their amino acid rich blood meal. Phenylpyruvate, the toxic accumulation product in human Phenylketonuria disease, was found to cause increased death when fed to mosquitoes. GC-MS analysis showed that similar to PKU patients, phenylpyruvate had been converted to another toxic metabolite phenyllactate. Using RNAi, two enzymes PAH and PPO9 of the phenylalanine pathway were knocked down and their effect on survival, behaviour and melanisation immune response was investigated. While knockdown of one enzyme caused a reduced melanisation in response to parasite infection, reduced activity of the other led to a shortened adult life span but no change in melanisation. This led to the conclusion that both enzymes are regulating different processes and that melanisation is not necessary for mosquito survival and possibly mosquito immunity.

Another strategy was to express long stretches of polyglutamines that are responsible for the toxicity of several neurodegenerative diseases, including Huntington's disease. Because uniform CAG repeats containing *huntingtin* exon 1 were highly instable in vitro and in vivo, different N-terminal huntingtin fragments with alternating CAG/CAA repeats fused to EGFP were inserted by *attP/attB* integration system in the model system *Drosophila melanogaster* and in *Anopheles gambiae* to characterise polyglutamine length dependent aggregation formation in the optic lobe and its effect on survival and behaviour. In both species, the polyglutamine fragments of putative toxic length tended to form aggregates in the photoreceptor cells. This led to a significant reduced adult life span in flies but not in mosquitoes. The latter showed a change in their chromatic vision ability. Alternative promoters were characterised for future HD mosquito models and could help to understand the observed difference between flies and mosquitoes. Both strategies have a potential as promising late-life acting vector control tools that merit further exploration.

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List of Abbreviations

ACT- artemisinin-based combination therapies

ATP- adenosine triphosphate

attP- phage attachment site

attB- bacterial attachment site

attL- left attachment site

attR-right attachment site

CI-cytoplasmatic incompatibility

CTP- cytosine triphosphate

DDC- dopa decarboxylase

DDT- dichlorodiphenyltrichloroethane

EGFP- Enhanced green fluorescent protein

Ex1- exon 1

Fig.- Figure

G- generation

GC-MS- Gas Chromatography-Mass spectrometry

GMAP- Global Malaria Action Plan

GTP- guanosine triphosphate

HAP-2- *hapless*-2

HBV- hepatitis B virus

HD- Huntington's disease

HEG- Homing endonuclease

Htt- huntingtin

httN25QEGFP- N-terminal huntingtin fragment containing 25 CAG/CAA repeats which is fused to EGFP

httN97QEGFP- N-terminal huntingtin fragment containing 25 CAG/CAA repeats which is fused to EGFP

IRS- indoor residual spraying

IPT- intermittent preventive treatment

ITN- insecticide treated bed nets

KEGG- Kyoto Encyclopaedia of Genes and Genomes

LLA- Late- Life Acting

LSA- Liver stage antigens

NTP- nucleoside triphosphate

RACE- Rapid Amplification of cDNA Ends

PAH- phenylalanine hydroxylase

PKU- Phenylketonuria

polyQ- polyglutamine

PPO9- prophenoloxidase 9

Q- glutamine

qRT-PCR- quantitative Real- Time PCR

RBM- Roll Back Malaria Initiative

RIDL- Release of Insects carrying a Dominant Lethal

RNAi- RNA interference

RTS,S- central repeat (‘R’ in RTS, S) and the entire carboxyl terminus (T) of CSP fused to the hepatitis B virus (HBV) surface (S) antigen (forming RTS) particles formulated in various adjuvants like ASO1 (S).

SIT- Sterile Insect technique

SP- Sulphadoxine–pyrimethamine

TH- tyrosine hydroxylase

TRAP- thrombospondin-related adhesion protein

TTP- thiamine triphosphate

TYR- tyrosinase

WHO- World Health Organisation

1. Chapter: Introduction

1.1. Malaria: situation and trends

In 2008, the World Health Assembly and Roll Back Malaria Partnership set the goal to reduce the number of malaria cases and deaths recorded in 2000 by 75% or more by 2015 (RBM, 2008) Although, due to malaria control interventions in the last 10 years, human malaria cases have dropped from 244 to 225 million per year and the number of deaths overall dropped from 985000 in 2000 to 781000 in 2009 the set goal will probably not be achieved (WHO, 2010). This means that malaria remains one of the 10 major disease burdens of human society (Lopez et al., 2006). There is even evidence that malaria increased in some countries in the last years, the reasons for the resurgence are not known for certain, but this fact highlights the fragility of malaria control (WHO, 2010).

In the next years all malaria infested countries will have to face future challenges: a) insecticide treated bed nets (ITNs) that have been distributed to the Sub-Saharan Africa will need to be replaced; b) more ITNs will have to be delivered to a growing African population; c) mosquitoes will potentially develop a widespread resistance to pyrethroids, which today are the single class of insecticides used in malaria countries and d) the continuous use of oral artemisinin-based monotherapies in some countries will foster the development of resistance to artemisinins which threatens the efficiency of artemisinin-based combination therapies (ACTs) (WHO, 2010).

Therefore, even if the number of malaria cases has dropped substantially, in order to promote and to maintain the downward trend in malaria, new alternative and effective intervention strategies need to be developed.

1.2. **The biology of human malaria**

1.2.1. **The parasite life cycle**

Human malaria is caused by five different protozoan parasites of the genus *Plasmodium*: *P. falciparum, P. ovale, P. malariae, P. knowlesi* or *P. vivax* which differ morphologically, in their relapse patterns and course of disease they cause as well as in their geographical distribution. They are all transmitted obligatorily from one human to another with repeated blood meals by female mosquitoes of the *Anopheles* genus (Cox-Singh and Singh, 2008).

The *Plasmodium* life cycle is complex and involves an asexual phase inside its human host as well as a sexual development that starts inside the human host but is finished inside its *Anopheles* vector (**Figure 1.1**). Once *Plasmodium* sporozoites are injected by a female *Anopheles* mosquito into the human blood they invade liver cells (hepatocytes) and differentiate into schizonts which develop new invasive motile forms, called merozoites (Sturm et al., 2006). After merozoites are released from the liver they invade red blood cells (erythrocytes) and continue their asexual reproduction followed by a periodical release of merozoites and reinfection of erythrocytes. Some *P. ovale* and *P. vivax* sporozoites can delay their formation of exo-erythrocytic phase merozoites by undergoing a non-infectious hypnozoite stage which lasts typically six months to up to three years (Cogswell, 1992). Merozoites enter the sexual stage by developing into gametocytes, which can be ingested with a mosquito blood meal and differentiate into male and female gametes within the mosquito midgut (microgametes and macrogametes). After fertilization of the gametes, the resulting diploid zygotes transform into motile haploid ookinetes which penetrate the plasma membrane and the midgut epithelium within 24h (Ghosh et al., 2000). They then form sporozoite- producing oocysts in the extracellular space between the midgut epithelium and the overlaying basal lamina. Oocysts rupture 10-24 days post infection, depending on the *Plasmodium* species, releasing thousands of sporozoites per oocyst in the hemocoel from which they invade cells of the salivary gland epithelium (Ghosh et al., 2000). In the salivary gland the sporozoites await introduction into a host during blood feeding. Once a female mosquito is infective, she remains so for life.

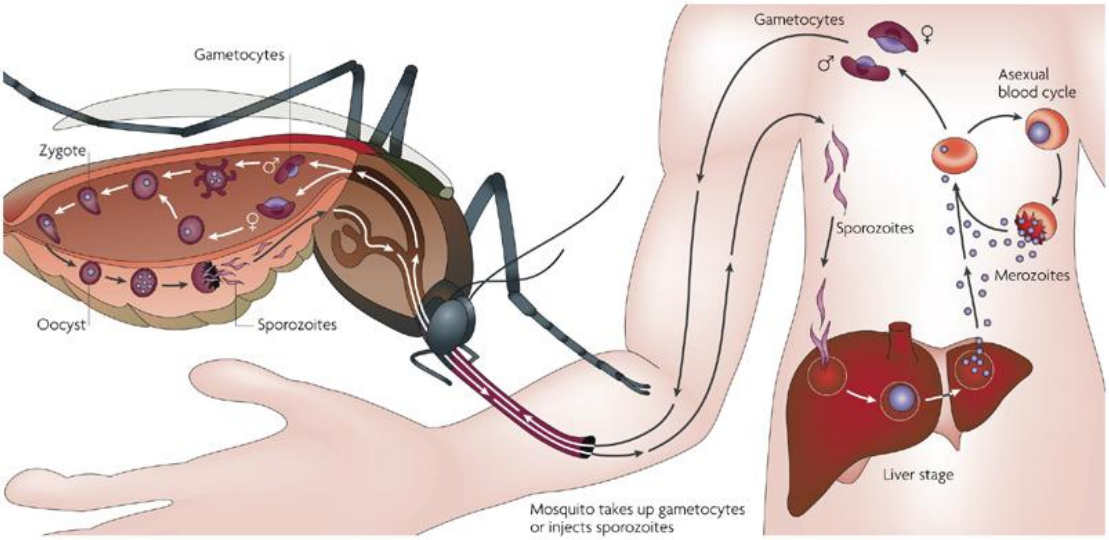


Fig 1.1: The *Plasmodium* life cycle (from Su et al., 2007).

1.2.2. **The vector species**

All vectors of human malaria belong to the genus *Anopheles*. There are 34 regionally dominant malaria transmitting *Anopheles* species in 260 endemic regions worldwide (Kiszewski et al., 2004). 90% of the Malaria cases occur in sub-Saharan Africa where it is more robustly transmitted than elsewhere in the world (**Figure 1.2**) (Breman, 2001). Especially in the Savannah- areas this is due to a continuous heat, high human biting rate of the autochthonous vector and the presence of a complementary vector (*An. funestus*) that maintains the transmission during the dry season when the density of the principal vector *An. gambiae* is low (Kiszewski et al., 2004). Its high relevance as malaria vector, makes *An. gambiae* a very suitable subject of this thesis. Interventions in *An. gambiae* populations are likely to have the highest impact on malaria transmission in these endemic countries.

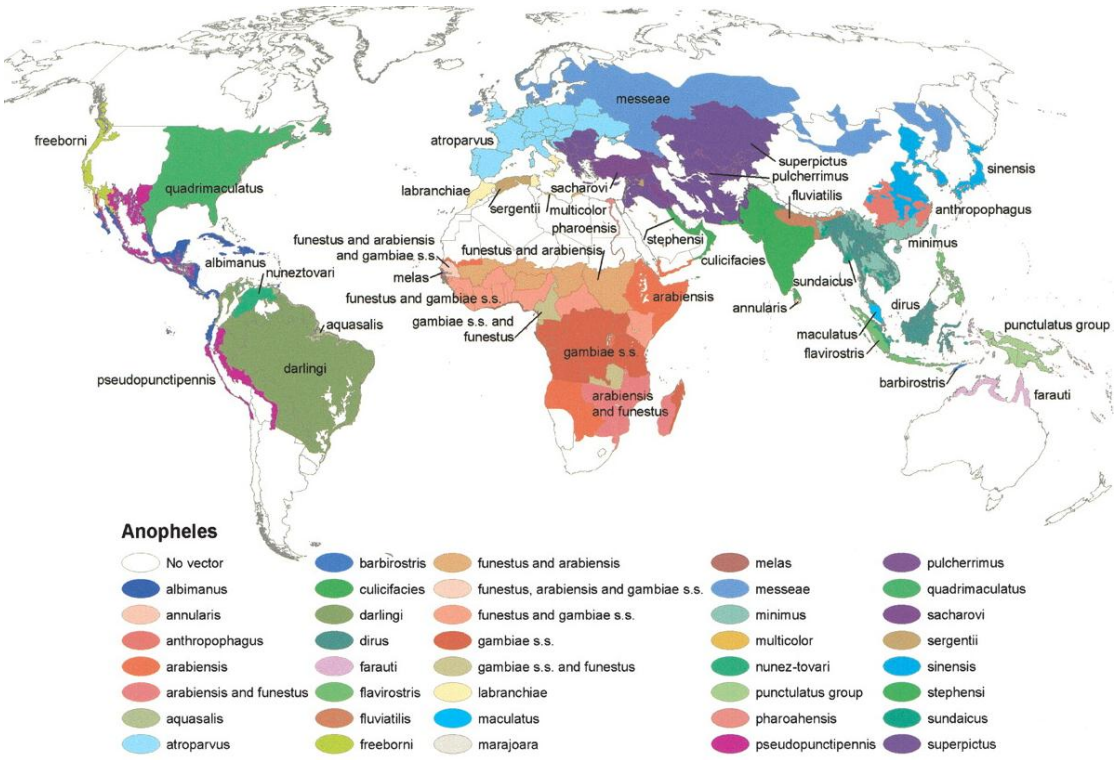


Fig. 1.2: Global distribution of dominant or potentially important malaria vectors. Selected are those *Anopheles* species that were longest lived and that fed most frequently on human hosts (Kiszewski et al., 2004).

1.2.3. **Importance of vector life span for malaria transmission**

The mosquito undergoes four larval stages and one pupal stage which can last 10-14 days before it emerges into adulthood. Within 24h after emergence female adults are able to mate and to ingest a blood meal which is needed for the production of eggs. Two days after the blood meal the gravid female lays its eggs. The parasite which has been been potentially ingested with an infected blood meal requires a minimum of 10 days to replicate and disseminate inside the mosquito body. Because the mosquito survival coincides with this long extrinsic incubation period (EIP), vector life span is an important determinant for the parasites ability to transmit infectious sporozoites into its human host (Gilles and Warrel, 2002).

The measurement of the direct adult survival of these females in nature is challenging as mosquitoes disperse far away from their breeding places to seek a host for blood

meals. Often this allows only estimates of the population size and survival rate. One widespread method of measuring the survival rate is the mark capture release method. Firstly, mosquitoes are collected either from laboratory colonies or from the field. The collected mosquitoes are then marked, released into the field, and recaptured at given time and distance intervals after their release. The ratio of recaptured marked versus non-marked insects allows an estimate of the population size and survival rate. Another strategy is to measure indirectly the survival of wild mosquitoes by counting the number of beadlike dilatations on the ovarioles which are formed with each gonotrophic cycle (Clements and Paterson, 1981, Detinova, 1962, Gillies and Wilkes, 1965, Davidson, 1954). More recently, a new approach for age determinations is to measure age related differences in gene expression in *Anopheles gambiae* (Cook and Sinkins, 2010).

By correlating the physiological age which was determined by the mark capture release with the number of gonotrophic cycles of wild *An. gambiae* mosquitoes it has been estimated that only 17% of females survived 3 gonotrophic cycles to reach an age of about 12 days that would allow formation of infectious sporozoites (Gillies and Wilkes, 1965). One reason for this low percentage could be that with increasing age most mosquitoes had dispersed. However, the fact that it coincides with the small proportion of 11-13% of wild caught mosquitoes which carried sporozoites suggests that in nature malaria is transmitted only by a small percentage of mainly old mosquitoes.

In this line malaria control models such as the model of the quantification of the basic reproduction number (R_0) by Macdonald (1957) and the concept of the vectorial capacity have highlighted the importance of the adult mosquito life span on the malaria transmission potential (Macdonald, 1957, Garrett-Jones, 1964). R_0 is defined as the expected number of hosts after one generation of the parasite by a single infectious person in a completely susceptible population; the vectorial capacity is the daily rate at which future human inoculations arise from a single infective case, describes more the entomological component of the Macdonald models by incorporating various factors such as the human biting rate, vector life span, human preference and vector density (Smith et al., 2007, Dietz, 1993) (**Figure 1.3**). This model shows that vectorial capacity increases linearly with the vector competence (ability to acquire and transmit parasite) and vector density, but increases

exponentially with the mosquito longevity (Cook et al., 2008). Consequently, when considering a vector population, its life span is the most sensitive determinant of capacity for pathogen transmission and therefore very suitable target for vector control. This seems to be confirmed by the fact that the most effective vector control strategies today are insecticide interventions like indoor residual insecticide sprays (IRSs) and long-lasting insecticide-treated nets (LLINs) that focus on the reduction of the survival rate of adult *Anopheles* vectors, (Enayati and Hemingway, 2010).

However, there is growing insecticide resistance and failure of other malaria control strategies by development of resistance of *Plasmodium* to drugs and lack of a vaccine show that these vector control strategies will need to be improved and replaced by new approaches.

$$V = \frac{ma^2 p^n b}{-\ln p}$$

where:
m = vector density in relation to host density
a = probability a vector feeds on a host in 1 day (= host preference x feeding frequency)
p = probability of vector surviving 1 day
n = duration of extrinsic incubation period (EIP) in days
b = vector competence
$1/(-\ln p)$ = duration of a vector's life, in days after surviving EIP.

Fig 1.3: The vectorial capacity (Garrett-Jones, 1964).

1.3. **Malaria control strategies**

The recent availability of the genome sequences of humans, *Anopheles* mosquitoes and the *Plasmodium* parasites offers possibilities for new interventions (Holt et al., 2002, Gardner et al., 2002). An overview of existing and potential new control measures is given below.

1.3.1. **Anti-malarial drugs**

There has been a long history over 100 years of the use of anti-malarial drugs to prevent malaria transmission in endemic areas. It has progressed from sole treatment of symptomatic cases and mass drug administration involving the whole population through sustained chemoprophylaxis targeting risk groups to intermittent preventive treatment (IPT) in which drug administration is reduced to the minimal level required to achieve a useful protective effect (Greenwood, 2004). Today the various anti-malarial drugs in use include quinolines, artemisinins and other combination drugs (Sulphadoxine–Pyrimethamine, Atovaquone– Proguanil, Lumefantrine– artemether). Synthetic aminoquinolines (Chloroquine, Amidoquine, Primaquine, Mefloquine) are effective against all forms of schizont and gametocytes of *Plasmodium* by inhibiting haemoglobin digestion (Foley and Tilley, 1997). Because of its high efficiency, low costs and relative safety chloroquine became quickly the most widely use anti-malarial drug in the past. However, due to its heavy use over decades resistant *Plasmodium* strains have spread and can be found worldwide (Payne, 1987, Peters, 1987, Rieckmann et al., 1989). Today, amidoquine, mefloquine and halofantrine are used against chloroquine resistant strains. However, there is also a high risk of development of resistance and medical risk associated with them (Wellems and Plowe, 2001, Dorsey et al., 2001) (WHO, 2001). Other drugs facing similar problems include Sulphadoxine–pyrimethamine (SP) combination drugs which act synergistically by inhibiting the dihydrofolate reductase and dihydropteroate synthase enzymes, depriving the parasite of essential folate cofactors and artemisinins and its semi-synthetic derivates (artemether, arteether and artesunate) (Ridley, 2002, Takechi et al., 2001). The latter is the preferred drug in endemic countries and acts by interfering with the parasite calcium regulation (Eckstein-Ludwig et al., 2003). Often it is used with longer half-life drugs to reduce treatment time and increase individual compliance (Nosten et al., 2000, Barnes et al., 2005). Further, it is anticipated that the rapid clearance of the parasites by artemisinin derivates will reduce the development of resistance to the partner drugs. However, the continuous use of artemisinin monotherapies threatens the use of ACT by fostering the spread of resistance to artemisinins. There have been first reports of development of resistance at the Thai-Cambodian border, where artemisinin monotherapies have been used for over 30 years which could soon spread worldwide (Dondorp et al., 2009). This is supported by

the fact, that by the end of 2010, 25 mainly African countries were still marketing artemisinin products for monotherapeutic use (WHO, 2010). A spread of artemisinin resistance seems therefore inevitable. Further, using ACT's with ineffective partner medicines will also increase the risk of development and spread of artemisinin resistance.

1.3.2. **Vaccines**

Thus far, there is no effective malaria vaccine. Reasons for this are a complex parasite life- cycle, antigenic variation and low understanding of the interaction between the parasite and the immune system of its host (Moran et al., 2009, Gardiner et al., 2000, Florens et al., 2002, Scherf et al., 2008, Langhorne et al., 2008). However, there are some promising malaria vaccine candidates that target the malaria parasites at the clinically silent sporozoite and liver stages of infection. One example is RTS,S which consists partially of a C-terminal fragment of the parasite-specific circumsporozoite protein (CSP) and is currently under evaluation in phase III clinical trials (Casares et al., 2010, Polhemus et al., 2009, Sacarlal et al., 2009, Guinovart et al., 2009). First results have shown a 50% reduction in the incidence of malaria in young children (Agnandji et al., 2011). There are efforts to improve the efficacy of CSP based vaccines with alternative adjuvants or viral vectors (Genton et al., 2010, Birkett et al., 2002, Felnerova et al., 2004, Walther et al., 2005, Walther, 2006). Another anti-sporozoite approach includes the irradiation or genetic attenuation of sporozoites or the expression of liver stage parasite proteins in viral vectors (Hoffman et al., 2010, Vaughan et al., 2010, Hill et al., 2010). In humans immunisation with viral vectors containing thrombospondin-related adhesion protein (TRAP) peptides led to a partial protection from *P. falciparum* infected mosquitoes (Webster et al., 2005).

The blood stage of the parasite life cycle, which begins with the release of merozoites from infected hepatocytes is the only disease causing period during the parasites life cycle and is another target for vaccines (Fairley, 1947). It is based on observations which showed in some people that repeated infections with the parasite led to blood-stage immunity to human malaria (Crompton et al., 2010). Antibodies play a key role for this acquired immunity. It is thought that by mimicking repeated parasite infection

through vaccination with blood stage antigens the naturally acquired immunity can be accelerated. Blood stage parasite antigens targeted in vaccine development include the apical membrane antigen 1 (AMA1), erythrocyte-binding antigen-175 (EBA-175), glutamate-rich protein (GLURP), merozoite surface protein (MSP) and serine-repeat antigen 5 (SERA5) (Sagara et al., 2009, El Sahly et al., 2010, Esen et al., 2009, Hermsen et al., 2007, Hill, 2011, Ellis et al., 2010). The main hurdle for the development of an efficient blood-stage vaccine will remain the genetic diversity of the parasite due to the selection pressure exerted by human immune responses (Weedall and Conway, 2010, Takala and Plowe, 2009).

A third vaccine strategy is based on the development of antibodies that are ingested with the mosquito blood meal in order to target the developmental stages of the parasite inside the mosquito midgut (Carter et al., 2000). This strategy is supported by the findings of transmission blocking antibodies in individuals living in endemic countries (Bousema et al., 2006). Examples of *P. falciparum* antigens expressed only in the mosquito are Pfs25, Pfs 48/45, Pfs230 and HAP-2 (Kaslow et al., 1989, Chowdhury et al., 2009, Liu et al., 2008). Previously, HAP-2 has been shown to induce strong malaria transmission blocking immunity (Blagborough and Sinden, 2009). However, in order to protect the individuals and block malaria transmission it is believed that these vaccines would have to be combined with other type of vaccines in an entire population (Crompton et al., 2010). Further, in intense malaria areas transmission blocking vaccines are not predicted to be effective unless they are combined with other vector control measures such as IRS or ITN (Carter et al., 2000).

1.4. **Vector control**

Vector control has been very successful in malaria control in various parts of the world; therefore renewed efforts in this field should play an essential role for the new malaria eradication strategy (Takken and Knols, 2009). The most successful and promising future vector control tools include biological and chemical control measures, environmental management and the application of transgenic technologies.

1.4.1. **Environmental management**

Although interest in environmental management strategies waned since the rise of DDT in the 1940s, the practise is now being reconsidered by countries looking for more sustainable, less pesticide-intensive approaches to malaria vector control (Walker and Lynch, 2007). The techniques include environmental modification/ manipulation and modification of human habitations/ behaviours (WHO., 1982; (Ault, 1994). Draining of wetlands, elimination or reduction of vector breeding habitats and house screens have successfully been used to control vector populations (Fantini, 1998, Karanja et al., 1994, Konradsen et al., 2004, Utzinger et al., 2001, Killeen et al., 2002, De Castro et al., 2004). However, there are risks associated with this strategy as poorly maintained drainage projects may actually increase larval breeding habitats (De Castro et al., 2004). Further, the wide array of vectors and their diverse habitat preferences make this strategy impracticable (Ramirez et al., 2009). Therefore, the efficiency of this approach depends on the adaptation of the available tools to habitat preferences of the local vector species and the local environmental conditions as well as the integration into agricultural practices and coordination on a local and regional level (Walker and Lynch, 2007, Lacey and Lacey, 1990).

1.4.2. **Chemical control by ITN and ITS**

In the past, the widespread use of DDT through indoor residual spraying (IRS) which targeted specifically adult mosquitoes in urban areas led to a reduction of mosquito vectors worldwide (Ramirez et al., 2009). However, within a short period of time, the development of DDT- resistant mosquitoes and chloroquine-resistant parasites led to a resurgence of malaria in many countries and included epidemics in some countries (WHO, 1969).

Today, the most effective vector control strategies are Long-lasting Insecticide treated nets (LLIN) and IRS. Both rely on the anthropophilic feeding behaviour of the mosquito and the reduction of the malaria transmission by shortening of the adult mosquito life span (Ramirez et al., 2009, Shiff, 2002).

However, these highly successful malaria vector control methods depend highly on a single class of insecticides, the pyrethroids, which boost development of resistance.

So far widespread examples of resistant *An. gambiae* mosquitoes have been reported in western Africa (Chandre et al., 1999, N'Guessan et al., 2007, Trape et al., 2011). Because of a high coverage of the use of pyrethroids and a high malaria burden, this resistance will soon spread throughout Africa, (World Malaria Report, 2010). Therefore, there is an urgent need to develop a new class of insecticides or other vector control interventions in order to sustain achieved reduction in mosquito prevalence in these endemic countries.

In 2009, Koella pointed out that the current WHO pesticide evolution scheme is outdated, because it leads to effective insecticides that act immediately and thus favour high selection pressure towards resistance (Koella et al., 2009). Killing sensitive mosquitoes as young adults after one bite, means such a high selection pressure, that only resistant mosquitoes would survive, which would lead to 100% resistance in the next generation (Koella et al., 2009).

Therefore, in order to reduce the selection pressure towards development of resistance new strategies need to be developed in order to kill disproportionally older mosquitoes after they have reproduced, but before they develop mature sporozoites. Killing older mosquitoes has also the advantage that the expression of key enzymes like acetylcholine esterase needed for the development of resistance decrease with age (Koella et al., 2009).

One approach is to develop late-life acting insecticides (LLA) that are effective against malaria transmission but yet generate little selection pressure for resistance.

Models have predicted that for example assuming a gonotrophic cycle every 2-3 days, killing mosquitoes at the fourth gonotrophic cycle would eliminate only 22% of the progeny, compared to 85% kill on first contact insecticides and still inhibit malaria transmission (Read et al., 2009, Gillies and Wilkes, 1965). Further, the inclusion of even modest costs of resistance would substantially slow the rate at which resistance to LLA insecticides spreads in a population, thus considerably prolonging the effectiveness of malaria control.

These LLA would not have to be conventional chemical insecticides. *Anopheles* mosquitoes can be infected with pathogens that shorten their life span. Examples are given in the next section.

1.4.3. **Biological control**

This strategy is used to control the vector population by predation, parasitism or competition. Effective biological control agents that have been employed against *Anopheles* larval stages are predatory fish and bacteria such as *Bacillus thuringiensis var. israelensis* as well as *Bacillus sphaericus* (Fillinger et al., 2003, Russell et al., 2003). These latter microbial agents function as stomach poisons in the mosquito larval midgut and have been successfully applied in rural western Kenya where they reduced the larval density by 95% (Fillinger and Lindsay, 2006).

Other microbial agents such as densoviruses, entomopathogenic fungi like *Metarhizium anisopliae* or *Beauveria bassiana* and the microsporidian *Vavraia culicis* have been used to increase adult mortality of *Anopheles* vectors (Carlson et al., 2006, Scholte et al., 2006, Blanford et al., 2005, Lorenz and Koella, 2011). Attempts to stably artificially-transinfect Anopheline mosquitoes with the intracellular bacteria *Wolbachia* has been so far unsuccessful (Hughes et al., 2011). However, *Wolbachia* has been shown to shorten the life span in *D. melanogaster* and *Aedes aegypti* and could potentially do so in Anopheline mosquitoes (Min and Benzer, 1997b, McMeniman et al., 2008, McMeniman et al., 2009).

In contrast to conventional chemical insecticides, however, these microbial parasites which replicate throughout the mosquito have the advantage that they increase in toxicity with age of the mosquito, leaving young mosquitoes less affected and able to lay eggs. These attributes could make these late-life acting biopesticides evolutionary sustainable alternatives to malaria control (Lorenz and Koella, 2011).

Although, these biological vector control measures have the potential for reducing *Plasmodium* transmission, there are hurdles related to the agent's efficiency which depends on environmental conditions, the density of the agent, the agent's specificity as well as the potential risk of development of resistance (Scholte et al., 2004, Kanzok and Jacobs-Lorena, 2006, Jiggins and Kim, 2005).

One alternative that is species-specific and has a relatively low impact on the environment is the Sterile Insect Technique (SIT). It involves the mass rearing and release of large number of sterile males which upon mating with the native females, result in unviable offspring and thereby cause a decline in the native population

(Thomas et al., 2000, Ramirez et al., 2009, Knipling, 1959). SIT programs have been used to control agricultural pests such as the New World Screwworm, *Cochliomyia hominivorax* in Central America, the Mediterranean fruit fly, *Ceratitis capitata* in Latin America and the tsetse vector of cattle trypanosomiasis, *Glossina austeni* in Zanzibar (Wyss, 2000, Vreysen et al., 2000). The most successful SIT programme as malaria control measure involved the release of chemosterilized *An. albimanus* males to suppress a local mosquito population in El Salvador (Lofgren et al., 1974). However, reasons for why SIT has not been widely applied to control mosquito populations involve the loss of male fitness after sterilisation by irradiation or chemosterilisation, the need to produce sufficient numbers of sterile males and the challenges posed by the biology of the mosquito population (Phuc et al., 2007). Recently, transgenic approaches have been used sterilising mosquito populations and are currently being developed to overcome the mentioned problems and improve the efficiency of SIT (Alphey, 2002, Andreasen and Curtis, 2005). This novel approach remains not limited to SIT and other possible applications to replace or to suppress a vector population are discussed below.

1.5. **Transgenic technologies as a new tool for vector control**

10 years ago, the sequenced genome of *Anopheles gambiae* and the successful germline transformation of *Anopheline* mosquitoes marked the beginning of a new era in understanding of the malaria-mosquito system and subsequently in vector control strategies that included the genetic modification of mosquitoes to suppress a target population or to replace it with mosquitoes refractory to the parasite (Holt et al., 2002, Catteruccia et al., 2000, Grossman et al., 2001).

1.5.1. **Population suppression**

The current transgenic strategies to suppress an Anopheline population involve transgenic SIT, the release of insects carrying a dominant lethal gene (RIDL) and transgene induced embryonic sterility of mosquitoes. Transgenesis could be used to make SIT more efficient by overcoming the mentioned obstacles of its fitness load on males and need for large numbers of sterile males. This fitness load could be for

example reduced by developing early stage sterility or by selective killing of females by expressing female specific lethal genes Such systems could be applied in RIDL programmes, which means the release of insects carrying a dominant lethal gene (Alphey and Andreasen, 2002, Thomas et al., 2000). In contrast to classical SIT the expression of female specific dominant lethal gene would not require the sexing and would be more efficient in reducing the mosquito population (Alphey et al., 2002). Homozygous males would mate with females; the resulting female progeny would die and reduce the reproductive capacity of the population. Then the now heterozygous male offspring would further reduce the population which is not the case for classical SIT.

Another strategy, to reduce the male fitness load is to genetically induce embryonic sterility (Horn and Wimmer, 2003). This system relies on mass-releasing homozygous males carrying a conditional lethal factor that is active in early embryos. Recently, complete and dominant embryo lethality has been generated successfully in *An. gambiae* (Windbichler et al., 2008). Heterozygous males that expressed an X-chromosome cutting enzyme during spermatogenesis were crossed with wild type females which led to an early death of embryos by shredding the maternally inherited X-chromosome (Windbichler et al., 2008). One obstacle of this approach is that there is no inherent sexing component with such transgene-induced embryonic lethality and it would therefore have to be combined with other existing sexing mechanisms.

1.5.2. **Population replacement**

Until recently, all vector control strategies worked on imposing negative effects on the vector population, but what is really important is to limit the transmission of the parasite (Michalakis and Renaud, 2009). Therefore an ideal approach would minimise the fitness load to the vector, to avoid the development of resistance, but harm as much as possible the pathogen. Genetically modified mosquitoes (GMM) could express anti-parasite effector genes through a suitable promoter which would make them impaired for malaria transmission. By means of a gene driving mechanism these GMM could then potentially replace native mosquito population.

1.5.2.1. **Population replacement using gene drive systems**

Gene drive systems are essential for the implementation of genetically modified mosquitoes as tool for malaria control. They could spread anti-parasite effector genes in a super Mendelian fashion which would allow more than 50% of the mosquito progeny to inherit the gene of interest leading to a more rapid fixation and less chance of development of resistance against this gene within a mosquito population.

Transposable elements (TE) such as *Hermes*, *Minos*, *Mos1* and *piggyBac* are mobile elements which can move rapidly into populations and have been essential for the development of transgenic technologies, but because of their low rate of transposition, they are currently not sufficient to serve as a drive system (O'Brochta et al., 2003). While TEs randomly integrate into a genome, HEGs cause a double strand break at a highly specific site lacking HEG and homologous repair by copying the HEG to the cut chromosome (Rong and Golic, 2003). Recently, HEGs have been successfully introduced and invaded naive *An. gambiae* populations (Windbichler et al., 2011).

Another gene drive mechanism for GMMs is the maternal-effect-dominant embryonic arrest or "medea" system. This approach is based on the maternal expression of a toxin by a germline-specific promoter that limits the survival to zygotes which express an antidote to this toxin. So far this system has been shown to be effective in fruit flies (Chen et al., 2007).

Further, *Wolbachia* could be used for gene drive because it has been shown to manipulate the mosquito reproduction to their own advantage by various patterns of cytoplasmatic incompatibility (CI). In the case of unidirectional CI for example sperm from *Wolbachia* infected males are unable to complete fertilization of uninfected eggs, but eggs from infected females develop normally, giving infected females a frequency-dependent reproductive advantage (Sinkins, 2006).

1.5.2.2. **Manipulating the vectorial capacity**

Since the development of the parasite within the mosquito involves several transitions of environment, physiology and morphology, there are several strategies to manipulate the vectorial capacity using transgenic technologies. One approach would

be to express a molecule which would then bind and change the function of proteins important for the parasite (Nirmala and James, 2003). Such effector molecules are currently developed to target parasite surface proteins, proteins needed for tissue invasion, receptors of mosquito tissues or affect the mosquito immune response. Another alternative approach to reduce the vectorial capacity would be to knock out or overexpress genes which change the mosquito feeding preference or the life span of a mosquito thus affect parasite transmission.

1.5.2.2.1. **Interfering with mosquito tissue recognition by the parasite**

Single chain antibody fragments (scFv) targeting the parasite surface proteins or receptors of invadable mosquito tissues have been able to inhibit successfully the parasite development in Anopheline mosquitoes. Examples for the targeted parasite surface proteins are the circumsporozoite protein , the *P. falciparum* chitinase 1 and the zygote and ookinete surface protein Pbs21 and Pfs25 (Li et al., 2005, Li et al., 2004, Barr et al., 1991, Yoshida et al., 2001, Isaacs et al., 2011). Other effector molecules targeting receptors of *Anopheles spp.* salivary gland or midgut tissues include SM1, LANB2 (laminin γ1) and the snake venom/ Bee phospholipase A2 (PLA2) (Ito et al., 2002, Moreira et al., 2002, Abraham et al., 2005, Zieler et al., 2001, Ghosh et al., 2009, Arrighi et al., 2005).

1.5.2.2.2. **Immune response effectors**

Possibly, due to a lower selection pressure upon *Plasmodium* infection and seasonal restriction of the parasite, the mosquitoes' immunity pathways have developed as antimicrobial defence against bacteria and fungi and not specifically against *Plasmodium* (Dong et al., 2006, Richman et al., 1996, Lowenberger et al., 1999). Already in 1989 Gwadz found that from the skin of frogs or from giant silk moths respectively derived antimicrobial agents magainins and cecropins were able to activate the *Anopheles* immune system against infections of various *Plasmodium* species (Gwadz et al., 1989). This result was later confirmed in a transgenic *Anopheline* model where *cecropin A* was expressed by a blood-meal inducible

promoter and negatively affected the development of *Plasmodium* parasites (Kim et al., 2004a).

Various studies have investigated the pathways involved in the immune response by using RNAi. In *Anopheles gambiae* mosquitoes, the knockdown of enzymes like the parasite binding thioester containing protein TEP1, leucine-rich-repeat immune protein LRIM1 and the cell-cell adhesion mediating type C lectins CTL-4 and CTLMA2 resulted in an increase in developing parasites and showed that they are important for the mosquitoes' innate immunity (Blandin et al., 2004, Osta et al., 2004). So far these studies are limited to RNAi knockdown because no gene targeting tool exists for mosquitoes. However, they give an insight into the mosquito defence mechanisms and provide future potential targets.

1.5.2.2.3. **Manipulation of the vectors' feeding preference**

The mosquito requires olfaction for its feeding, host preference and mate selection. Targeting olfactory pathways could therefore be another potential effector mechanism to reduce malaria transmission. So far 24 odorant receptors (AgOR) have been identified in *An. gambiae* which respond to a wide range of odorant stimuli with particular affinity for heterocyclics and aromatics that are associated with human skin emanations (Cork and Park, 1996, Bernier et al., 2000, Kwon et al., 2006, Carey et al., 2010, Wang et al., 2010). Gene silencing is currently used to determine the role of these AgORs and olfactory pathways in *Anopheles gambiae* (Liu et al., 2010).

1.5.2.2.4. **Inducing late-life mortality**

Recent models on LLA and experiments involving biological pesticides have shown that malaria control strategies which cause increased adult mosquito mortality with age could potentially be effective for inhibiting malaria transmission whilst still allowing reproduction of young mosquitoes and thus reduce the selection pressure for development of resistance. However, there are some obstacles with this approach because so far no chemical LLA exist and the toxicity of biological LLA pesticides depends highly on the infective dose which affects their effectiveness (Ledermann et al., 2004). Further, these microbial agents are not only transmitted vertically but also

horizontally in larval habitats which could potentially lead to infection of non-target species (Carlson et al., 2006). Additionally, like residual insecticides, the gradual loss of viability of entomopathogenic fungal spores on treated surfaces over time will require repeated application for sustained disease control (Scholte et al., 2005).

One alternative species- specific strategy to accelerate late-life mortality is the engineering of transgenic mosquitoes with a shortened adult life span and replacement of natural population by using a suitable gene drive system.

A very recent study has shown that by transgenic overexpression of AKT, a key component of the insulin-signalling cascade (IIS), the survival of adult *Anopheles* mosquitoes could be reduced by 20%. (Corby-Harris et al., 2010). Additionally, parasite oocyst formation was completely inhibited. This result was very promising for this new adult life shortening approach. Nevertheless, due to high number of substrates for AKT the exact mechanism by which AKT overexpression regulated innate immunity and life span is unknown and most likely other IIS regulated life traits such as stress response and fertility are affected by this transgene induction (Corby-Harris et al., 2010, Manning and Cantley, 2007).

In this project other potential life shortening strategies were investigated. However, identifying potential candidate genes was difficult as there are many gene mutations known to cause shortened life span in yeast, worms, mice, humans and flies but often these have pleiotropic effects reflecting their normal functions in cellular maintenance (cell cycle, repair, protein degradation); detoxification; lipid storage, vesicle transport or ATP synthesis (examples can be found in the **Appendix Table 10.1**). For this project this meant that a mutation of a gene in a mosquito would most likely cause, in addition to a shortened life span unwanted side-effects that reduce the fitness of the organism and its likelihood of success in a population replacement strategy. Bearing this in mind, the fitness cost could be potentially circumvented in combination with a strong gene drive system (Ribeiro and Kidwell, 1994, Hickey, 1982). Nevertheless, the efficiency of the transgene to cause late-life lethality would need to be very close to 100% to substantially decrease disease prevalence in high endemic areas (Boete and Koella, 2002).

1.5.2.2.4.1. **Inducing a blood-meal responsive amino acid disease**

Mosquitoes of the genus *Anopheles* are anautogeneous and require a host blood meal for their egg production (Okech et al., 2003). This blood meal creates a unique metabolic challenge for the adult female mosquito; extensive amounts of amino acids and proteins are ingested and need to be processed. Around 80% of the ingested proteins can be digested within a day (Lemos et al., 1996). It is known from vertebrate metabolism that if these ingested amino acids are not processed properly, over a period of time they accumulate progressively and form toxic metabolites which can lead to brain damage and mental retardation. Many of the enzymes involved in amino acid metabolism are conserved in mosquitoes, and indicate that the underlying metabolic pathways are regulating essential processes in life.

Therefore, one strategy in this project was to investigate whether similar toxic mechanism by which unprocessable metabolites accumulate due to a malfunctioning enzymes are conserved between humans and mosquitoes and whether they would lead to the mosquito's death.

1.5.2.2.4.1.1. **Phenylalanine/ tyrosine metabolism**

In humans as well as mosquitoes phenylalanine/ tyrosine metabolism is responsible for the formation of important neurotransmitters and melanin biosynthesis.

The key metabolite tyrosine is derived from phenylalanine that can be only obtained from digested food. Tyrosine is then converted to tyramine and octopamine (the invertebrate counterpart of adrenaline and noradrenaline) or 3-4 dihydroxyphenylalanine (Dopa), the precursor of dopamine or melanin (Roeder, 2005). In excess tyrosine gets degraded into water and carbon dioxide via 4 hydroxyphenylpyruvate and homogentisate into fumarate that is part of the Citrate Cycle (**Figure 1.4**). In humans this pathway is well studied because for each failing enzyme a human disease is described. One disease is Phenylketonuria (PKU), which is caused by mutations in the phenylalanine to tyrosine converting enzyme phenylalanine hydroxylase (PAH). A lack of PAH is associated with an accumulation of phenylalanine and its toxic transaminase products (phenylpyruvate, phenyllactate, phenylethylamine) that if left untreated leads to changes in energy metabolism,

cholesterol- and fatty acid synthesis, neurohormone and cerebral protein synthesis causing severe brain damage (**Table 1.1**) (Michals and Matalon, 1985, Bowden and McArthur, 1972, Patel, 1972, Silberberg, 1967, Land and Clark, 1973). Because mosquitoes ingest phenylalanine with their blood meal which is followed by its metabolisation into similar products as in humans, it was assumed that it could be feasible to mimic an amino acid disease like PKU by knockdown of the responsible enzymes in an *Anopheles* model. In other organisms like *Aedes* and *Drosophila* lack of PAH have been associated with reduced melanisation response upon infection with parasites, defects in eye pigmentation and even lethality in combination with a lack of Xanthine dehydrogenase ("rosy") which is involved in purine nucleotide synthesis (van Atta, 1932, Infanger et al., 2004, Lucchesi, 1968). All of these effects would be relevant for malaria transmission.

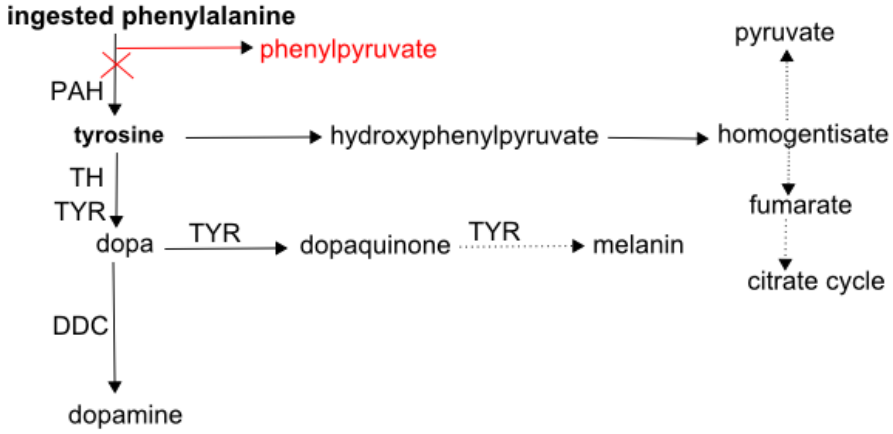


Fig 1.4: Brief overview of the phenylalanine metabolism. PAH- phenylalanine hydroxylase, TYR-tyrosinase, TH- tyrosine hydroxylase, DDC- dopa decarboxylase. The alternative phenylalanine pathway of Phenylketonuria patients is displayed in red.

Table 1.1 Enzymes inhibited by phenylalanine metabolites

enzyme	metabolism	inhibiting metabolite	reference
Pyruvate dehydrogenase	Lipid synthesis	phenylpyruvate	(Bowden and McArthur, 1972)
6-phosphogluconate dehydrogenase	Lipid synthesis	phenylpyruvate	(Patel, 1972)
NADP- Malate dehydrogenase	Lipid synthesis gluconeogenesis	phenylpyruvate	(Gimenez et al., 1977)
Citrate synthase	Lipid synthesis	phenylpyruvate	(Patel, 1972)
AcetylCoA carboxylase	Lipid synthesis	phenylpyruvate	(Land and Clark, 1973)
Fatty acid synthetase	Lipid synthesis	phenylpyruvate	(Land and Clark, 1973)
Hydroxybutyrate dehydrogenase	Lipid synthesis	phenypyruvate, phenylacetate	(Benavides et al., 1976, Gimenez et al., 1977)
3-oxo acid CoA-transferase	Lipid synthesis	phenylpyruvate	(Benavides et al., 1976)
3-hydroxy-3 methyl glutaryl coenzyme A	Cholesterol synthesis Coenzyme Q10	phenylalanine	(Williams et al., 2008)
Pyruvate decarboxylase	gluconeogenesis	phenylpyruvate	(Patel and Tilghman, 1973)
Lactate dehydrogenase	gluconeogenesis	phenylpyruvate	(Gimenez et al., 1977)
Hexo kinase	Glycolysis	phenylpyruvate	(Weber et al., 1970)
Pyruvate kinase	Glycolysis	phenylalanine, phenylpyruvate	(Weber et al., 1970) (Edwards and Blau, 1972)
Dopa decarboxylase	Catecholamine synthesis	Phenylpruvate, phenyllactate, phenylacetate	(Fellman, 1956, Boylen and Quastel, 1961)
5- Hydroxy-tryptophane decarboxylase	Catecholamine synthesis	phenylalanine, phenylpyruvate, phenyllactate, phenylacetate	

1.5.2.2.4.2. **Introducing a polyglutamine disease in *Anopheles gambiae* mosquitoes**

The induction of an amino acid disease in a transgenic model requires a gene targeting system that is not yet available for *An. gambiae*. As such, the expression of toxic proteins e.g. protein aggregation diseases such as Alzheimer, Parkinson or polyglutamine diseases that become toxic by degenerating neurons at adult stages and lead ultimately to death could be another option in a transgenic *Anopheles gambiae* model. The most common form of neurodegenerative disorders are polyglutamine diseases. There are 9 polyglutamine diseases Spinocerebellar ataxia (SCA) 1, 2, 3, 6, 7, 17, DRPLA, SBMA and Huntington's disease (HD) caused by single-gene mutations that lead to large unstable expansions of cytosine-adenine-guanine (CAG) repeats, which are translated as glutamine (Q) in functional unrelated proteins. Whereas a normal polyglutamine length has no pathological consequences, polyglutamine expansion beyond a critical threshold leads to a dominant inheritable toxic gain of function of these mutant proteins which form nuclear inclusions and lead to neuronal cell death (Kim et al., 2004b). An important feature of these disorders is that regardless of the type of polyglutamine disease, the longer the polyglutamine repeats the earlier the age of onset of the disease (Martindale et al., 1998, Trottier et al., 1994) **(Figure 1.5)**. This is advantageous because in contrast to other adult onset neurodegenerative diseases, this allows flexibility in modification of the onset of toxicity in the mosquito.

The best studied polyglutamine disorder is Huntington's disease which is caused by a polyglutamine expansion (Q) of over 35 repeats in the gene *huntingtin* (*htt*). This instability of the polyQ tract is due to hairpin formation which then leads to strand slippage during DNA replication (Petruska et al., 1998). Both gain-of-function (of the mutant protein) and loss-of-function (of the normal protein) mechanism contribute to the pathology of the disease, which include nuclear inclusions of mutant short N-terminal htt fragments, aberrant vesicle transport along axons, mitochondrial dysfunction, altered Calcium regulation, transcriptional dysregulation, inhibition of the ubiquitin-proteosome system and selective neurodegeneration (Zuccato et al., 2010).

Although this disease has been extensively studied it is still unclear whether the toxic agent is the expanded RNA or the protein and why certain neuron types are affected

more than others. Further, it is unknown whether the HD characteristic nuclear inclusions are toxic by binding ubiquitin which is needed for proteolysis or whether they are actually protective by accumulating short pathogenic N-terminal htt fragments (Zuccato et al., 2010, Weiss et al., 2011, Arrasate et al., 2004).

Htt is a very ancient gene which shares homology with htt genes of model organisms such as *D. melanogaster* and *C. elegans* and therefore might have a similar function (Kauffman et al., 2003, Sathasivam et al., 1997, Li et al., 1999, Parker et al., 2001, Zuccato et al., 2010). Overexpression of mutated human polyQ containing htt-exon1 in *Drosophila melanogaster* similarly shows repeat length toxicity and loss of photoreceptor neurons following expression and HD characteristic nuclear inclusion in the eye (Jackson et al., 1998, Marsh and Thompson, 2006, Marsh et al., 2000). The toxicity observed in the HD fly photoreceptor cells can be aggravated by panneuronal expression of pathogenic huntingtin (htt) causing pharate adult lethality (Weiss et al., 2011). Recently it also has been reported that human expanded N-terminal htt interacts with at least 27 endogenous *D. melanogaster* proteins which are mainly transcriptional regulators, suggesting that the action of huntingtin is functionally conserved (Kaltenbach et al., 2007).

A BLAST search and subsequential alignment revealed that the *Anopheles gambiae* protein AGAP003681 shares 28% sequence homology with the *Drosophila melanogaster* Htt protein (**Appendix Figure 10.1**) which supports the approach of expressing mutated expanded N-terminal htt to recapitulate a HD like disease in the malaria vector.

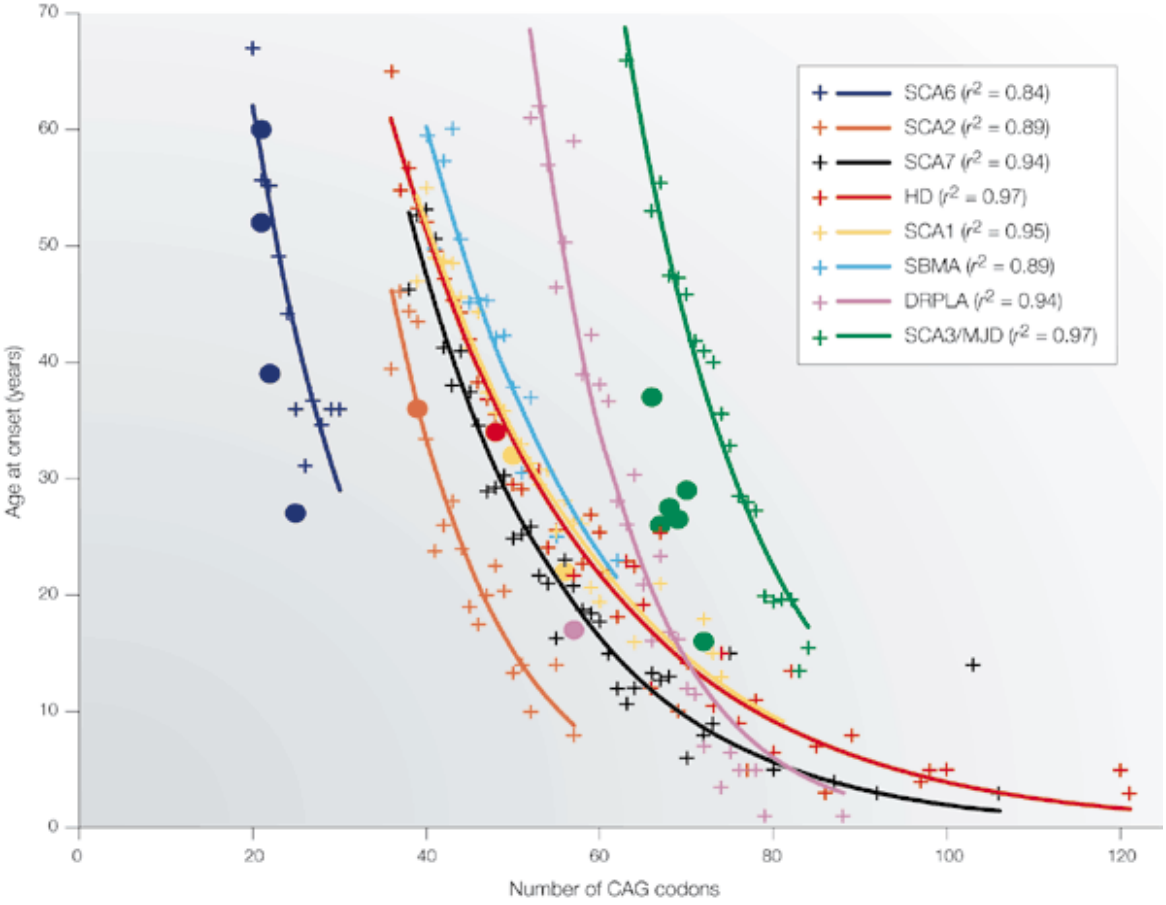


Fig. 1.5: Correlation between age of onset and number of CAG codons (polyQ) in various polyglutamine diseases. Data from the different references were used to calculate the mean age of onset (denoted by"+") associated with various CAG repeat length and the best-fit curve (smooth line) using an exponential decay model for the relationship, in each of the polyglutamine disorders (Gusella and MacDonald, 2000). It also shows the age of onset of homozygotes for the various disorders (filled circles), plotted accordingly to the longer of their two expanded CAG repeats. DRPLA- Dentatorulbropallidoluysian atrophy, HD- Huntington's disease; MJD- Machado-Joseph disease; SBMA- spinal and bulbar muscular atrophy, SCA- spinocerebellar ataxia.

1.6. **AIM**

The main aim in this project was to identify two new ways of accelerating late-life mortality in *Anopheles gambiae* through mimicking human diseases and further explore the effect on other parameters important for vectorial capacity.

The first objective was to test experimentally the toxic mechanism of the human disease PKU in mosquitoes by feeding its toxic accumulation product phenylpyruvate and measuring its effect on the mosquito survival. Based on the success of this experiment, the possibility of achieving such life shortening disease by knockdown of the disease causing malfunctioning enzyme phenylalanine hydroxylase in response to the mosquito blood meal was assessed. Subsequently, in order to establish the suitability as potential future target for gene-knockout technologies other effects on important life traits such as immunity, behaviour and reproduction which are important for malaria transmission were determined. Depending on the effectiveness of RNAi and phenotypic affect of these experiments other important dopamine and melanin rate-limiting enzymes of this phenylalanine pathway were targeted.

The second main objective was to produce transgenic animals carrying various lengths of htt fragments to examine their intrinsic mortality. The advantage of this approach was that the severity and age of onset of the disease depends on the expanded polyQ length of the huntingtin fragment, which means that in the future the disease could be timed so that the mosquitoes are able to reproduce at least once, but die before the parasite develops infectious sporozoites.

Having initially encountered problems with the stability of polyQ constructs, newly developed modified polyQ constructs were first validated in the more tractable model insect *D. melanogaster*. Based on success in this insect, the same polyQ constructs were examined in a transgenic *An. gambiae* model and effects on longevity and behaviour was assessed.

2. Chapter: Material and Methods

2.1. Bacterial Cultures

For *Escherichia coli* standard/ high efficient transformations DH5α™ or One shot Top10 cells (Invitrogen) were cultivated in Lysogeny broth (LB) medium (1% bacto-tryptone, 0.5% yeast extract, 1% NaCl). 4ml of this culture was then used for subsequent DNA preparation.

2.2. Isolation of plasmid DNA (Miniprep, Maxiprep)

For DNA manipulation procedures (e.g. cloning) the plasmid DNA of a 4ml overnight culture was purified using a Miniprep Kit (Qiagen). Large-scale DNA purifications of a 400ml culture, needed for the injection of plasmid DNA (~1µg) into the mosquito, were performed with Endofree Plasmid Maxi Kit (Qiagen).

2.3. Restriction digestion of DNA

All digests were performed in a 15-20µl volume at 37°C for 2h in the appropriate 1x buffer for the enzymes (Roche or NEB). In general 1 unit of enzyme is enough to digest 1µg of lambda DNA in 1h at its optimum conditions (temperature, buffer), here the enzyme was given 4 fold excess in order to achieve complete digestion. For subsequent cloning procedures the cut DNA plasmid fragments were dephosphorylated to prevent religation or separated on a 1% agarose gel followed by excision.

2.4. Dephosphorylation of cut plasmids

The enzyme, T4 DNA Ligase, used for ligation, requires 5' phosphorylated DNA strands to ligate DNA fragments. To prevent religation and to allow insertion of a fragment the cut vector plasmid was dephosphorylated by incubation with 1µl of Calf

Intestine Alkaline Phosphatase (CIP) (Roche) or Antarctic Phosphatase (NEB) for 30min. at 37°C. The Antarctic Phosphatase was heat-inactivated by incubating the reaction at 65°C for 20min, whereas the CIP was separated from the dephosphorylated DNA fragment by excising the DNA after separation on 1% agarose gel.

2.5. **Extraction of DNA from Agarose gels**

DNA fragments that were used as vectors or inserts for ligation were loaded on a 1% agarose gel (in 0.5x TAE buffer) containing SYBR Safe™ DNA gel stain, excised with a scalpel under a Safe imager™ blue light transilluminator and purified using QIAquick Gel Extraction Kit (Qiagen). After melting of the DNA containing agarose the nucleic acids are bound under high salt conditions to a silica membrane, followed by washes with 70% ethanol containing buffers to remove residual salts and elution with 30µl dH_2O. The concentration of the DNA that is needed for the followed ligation was determined with a Nanodrop Spectrophotometer at 260 and 280nm. Thereby at 260nm an OD of 1 unit corresponds to ~50µg/ml of DNA. The purity of the sample is represented by the 260/280nm ratio (high purity ~1.8).

2.6. **Ligation of plasmid DNA**

The following ligation reaction was performed in a volume of 10µl; containing the insert and vector at a molar ratio of 3:1 (~30ng vector) and the T4 ligase/ ligase buffer mix (Takara Bio Inc., Shiga, Japan) at 16°C for 1h. Thereby 5' phosphate and 3'-hydroxyl groups of vector and insert DNA were fused. Controls containing no ligase were used to check for the event of religation or incomplete digestion of the digested vector DNA.

2.7. **Transformation of *E. coli* with plasmid DNA**

All transformations were performed under heat shock. 50-100µl Competent $CaCl_2$ treated *E.coli* cells were thawed on ice for 30min after adding the ligation mix. The

cells were then heat-shocked for 30sec in a 42°C water bath to introduce the plasmid and stored for 2min on ice. After the cells were shaken in 250µl SOC medium they were plated on ampicillin containing agar. Colonies of cells carrying the inserted plasmid were cultivated in Luria Broth media containing ampicillin overnight at 37°C.

2.8. **Polymerase- chain reaction of plasmid DNA**

PCR was used to amplify a specific region of a DNA template using a Hybaid Thermal Cycler. The reaction contained: 5-25ng of template DNA, 0.5units of TAQ polymerase or Phusion Hot Star (Fermentas), 10mM dNTPs , 1x PCR MgCl2 buffer(10mM Tris-HCL, 1.5mM MgCl2, 50mM KCl, pH 8.3) and 50pmol of each primer (forward and reverse) in a final volume of 25ul. The TAQ Hot Star (Qiagen) was used to amplify plasmid DNA with a PCR program of 95°C 10min and 30-35 cycles of 95°C 30s, 50-58°C 30sec, 72°C 30s-1.30min and finally 72°C for 10min.

2.9. **Generation of the httex1pQ transformation vectors**

For the generation of the httex1pQ transformation vector, the eye- and neuron specific *3xP3* promoter and the *SV40* terminator fragment (containing a polyadenylation signal) were amplified from the PYSC 7 plasmid (Windbichler, unpublished, **Appendix Figure 10.2**) by polymerase chain reaction (PCR) using the proof-reading Phusion polymerase (NEB). This PYSC 7 transformation vector contains further an enhanced green fluorescent protein gene (*EGFP*) under regulation of *3xP3*, *ampicillin* resistance gene and an *attB* site that, in presence of the integrase enzyme, allows the site-specific integration of the gene cassette into the transgenic *attP* line E.

Restriction sites (underlined) for cloning were introduced into the primers used to amplify regions by PCR (*3xP3* fwd 5'-CGGGATCCGTCGACTCTCTAGCGGTAC CC-3' and *3xP3_Nco*I rev 5'-CATGCCATGGCGCACGGTTCCACAATGG-3', *SV40_BamH*I fwd 5'-GGAATTCCATATGAAGCGATGAGATCTGCTAGC-3' and *SV40* rev 5'-GCTCTAGATAGATCATAATCAGCCATACC-3'). Since the *3xP3* promoter contains an internal *Bam*H1 site the two fragments were inserted separately. The first 90bp of the *3xP3* containing the new restriction site *Nco*-I and a *Bam*HI site

were cloned into the shuttle vector pSLfa1180 (Horn and Wimmer, 2000) (**Appendix Figure 10.3**). This was followed by *Xba*-I/*Nde*-I insertion of *SV40*. Then *huntingtin* exon 1 fragments with 20, 51 and 93 polyglutamine repeats from PUAST plasmids (Steffan et al., 2001) (kindly provided by Leslie Thompson, University of Irvine) were *Bam*HI/ *Xba*-I integrated between the *3xP3* fragment and the *SV40* terminator. The second *3xP3* fragment of 176bp was cloned between the existing fragment of *3xP3* and the polyQ construct in the dephosphorylated *Bam*HI site of the shuttle vector. The final gene cassette was inserted as an *Asc*I fragment into the PYSC 7 transformation vector.

2.10. Generation of the *3xP3* httNQEGFP transformation vectors

The cloning strategy for the httNQEGFP transformation vectors was as follows: The donor pcDNA3.1 incl. CAG/CAA_{25} or $CAGCAA_{97}$ +EGFP (kindly provided by Leslie Thompson, University of Irvine) and the recipient PYSC7 plasmid were both digested with the restriction enzymes *SalI* and *XbaI*, which released the 1030bp htt-N-25Q-EGFP or 1240bp htt-N-97Q-EGFP fragment from the donor and the 810bp EGFP fragment from the recipient vector. In order to prevent methylation of the *XbaI* site of the PYSC7 vector (GATC recognition) dam^- cells (strain C2925 from NEB, chloramphenicol resistant) were used to propagate the PYSC7 plasmid. Then the *Sal*I-*Xba*I excised 2 htt fragments which were each ligated into a *SalI* and *XbaI* digested PYSC7 plasmid, which is located between the *3xP3* promoter and *SV40* terminator.

Due to the novelty of the introduction of the polyQEGFP fusion protein into the mosquito system, another marker construct *Actin-RFP-HST* from the pslfaActin5c(RFP)HST fa shuttle vector that has been used in mosquitoes before, was inserted into the *AscI* site.

2.11. **Generation of the *ELAV* httNQEGFP transformation vectors**

The subsequent cloning of the panneuronal *Dmelav* promoter into the httNQEGFP vectors was done as follows: Firstly, the httNQEGFP vectors prior to the *AscI-ActinRFP-AscI* marker insertion were cut with the restriction enzymes *AgeI* and *SalI* releasing the 262bp *3xP3* promoter. Then the *Dmelav* promoter was amplified from the PMO246 pattb-elav vector (kindly provided by Paul Overton, Oxford University) with primers with attached *AgeI* and *SalI* restriction sites (*ELAV* fwd 5'-ATACCGGTCCCGGATCCAGTCGAGG-3', *ELAV* rev 5'-CGGTCGACCCTATT GTTTTAGTTTAGGGAGCGAG-3') and inserted into the httNQEGFP vectors. This was then followed by cutting the vector with *AscI* and the insertion of the *SalI* site containing *AscI-ActinRFP-AscI* marker construct.

2.12. **Phenol/ Chloroform extraction**

1 volume of Phenol-chloroform solution (1:1) was added to 1µg linearised plasmid DNA in 200µl dH_2O, mixed by vortexing and spun down at 10000g for 5min. To the upper aqueous phase 1/ 10 volume of 3M sodium acetate and 2.5 volumes ethanol were added, followed by vortexing of the sample and incubation at -20ºC for > 10min. The pellet containing the DNA was recovered after centrifugation at 10000g for 15min. The plasmid DNA was then denatured in 200µl ethanol, centrifuged at 10000g for 5min and rehydrated in 20µl dH_2O.

2.13. **Synthesis of capped integrase RNA**

A capped Integrase RNA, mimicking most eukaryotic mRNAs was used to mediate germline transformations into the *attP* insertion sites of the *An. gambiae attP* line E and the *D. melanogaster* line *y1 w67c23; P{CaryPattP2* (Bloomington stock 8622).

In the first step 1µg purified pET11φC31polyA plasmid under transcriptional control of the *T7* promoter was digested with *BamH1* for 2h at 37°C to linearise the DNA. The DNA was then treated with 200µg/ml proteinase K and 0.5% SDS at 50°C for 30min. followed with Phenol/ Chloroform extraction to remove all proteins that might

be detrimental to further manipulations of the nucleic acid and ethanol precipitation. The pellet was then suspended in 20µl nuclease-free H_2O and stored at -20°C. For the synthesis of capped RNA, the mMessage mMachine Kit (Ambion) was used. 1µg linearised plasmid was added to a transcription reaction (20µl) containing 10µl 2x NTP/CAP (the reaction cap: GTP ratio is 4:1), 2µl 10x reaction buffer, 2µl enzyme mix (T7 polymerase buffered in 50% glycerol, RNase Inhibitor) and nuclease-free water. After the capped transcription reaction was incubated for 1hr at 37°C, all traces of DNA were removed by Dnase treatment followed by recovery of the RNA by lithium chloride precipitation. The capped integrase RNA was dissolved in 10µl dH2O. 1µl was used for a 10 fold dilution to measure the RNA concentration by spectrophotometry and to confirm the single band on an agarose gel.

2.14. **Mosquito strains**

Previous germline transformation in Anopheline mosquitoes relied upon transposable elements such as *Minos* or *piggyBac* that have a limited carrying capacity and insert transgenes essentially randomly (Grossman et al., 2001, Catteruccia et al., 2000). The latter can cause insertional mutagenesis and position effects. To circumvent these problems Meredith and colleagues applied the Streptomyces φC31 site-specific integrase system to *Anopheles gambiae* and obtained 4 independent strains (Meredith et al., 2011). The Anopheles gambiae (KIL) strain E which contained a *piggyBac*-mediated integration of the *attP* target site, linked to an ECFP marker under *3xP3* regulation at chromosome 3R 31B (position: 15801959) was used in this project for the generation of three different httex1pQ expressing lines. However, since the *3xP3* promoter/ *SV40*T cassette mediated ECFP as well as EGFP and Huntington exon1 expression there was a risk that recombination between the separate cassettes could cause the EGFP and polyQ to segregate leaving the polyQ lines without any marker.

In order to avoid this risk, a new *attP* docking line "X1" which had no visible marker but an attP site on chromosome 2L 21D (position: 10,184,850) (Naujoks and Marois, unpublished) was used for subsequent integration of the more stable httNQEGFP constructs. This "X1" line was obtained by *cre-lox* recombinase mediated excision of the enzyme cassette containing the *3xP3* RFP marker and enzymes I-SceI and FLP

from the “VFS-1” strain leaving the attP site and *piggyBac* inverted repeats from the original genomic integration (Naujoks and Marois unpublished).

The wild type *An. gambiae* strain (G3) was used for outcrossing of transient and transgenic mosquitoes as well as control, in addition to the newly established “X1” line, for the life span and behavioural assays.

2.15. **Mosquito rearing**

All strains were reared under standard conditions at ~27°C and 30-60% relative humidity with access to fish food as larvae and 10% glucose solution as adults. For egg production, young adult mosquitoes (3-5 days post-emergence) were allowed to mate for at least 2 days and then fed on mice. 3 days later an egg bowl containing rearing water (dH_2O supplemented with 0.1% salt) was placed in the cage. 1-2 days after hatching the larvae (L1 stage) were placed into rearing water containing trays (1 larva per litre) (www.MR4.org). ~50 Larvae were fed with 2 fish pellets (TetraPond) per day.

2.16. **Embryo microinjection in mosquitoes**

Blood-fed *An. gambiae* mosquitoes of the *attP* line E or X1 line were allowed to lay eggs on moist filter paper 72h after blood meal. 20min after egg laying ~40 eggs were aligned at a 45° angle on a glass slide and injected into the posterior pole with a micromanipulator (Narishige) at 100x magnification as previously described (www.MR4.org). The constructed httex1- 20Q, httex1-52Q and htt-69Q plasmids were coinjected with capped transcript of φC31 integrase, whereas the httN-25QEGFP and httN-97QEGFP constructs were injected with a plasmid that expressed the φC31 integrase under the germline specific *vasa* promoter (Papathanos et al., 2009) (the plasmid was kindly provided by Marois, unpublished). Usually a 10µl injection mix containing 1x microinjection buffer (50mM KCl, 1mM NaPO4), 300ng/µl transformation plasmid and 900ng/µl φC31 integrase RNA or 150ng/µl vasa-φC31 integrase plasmid was prepared and centrifuged to precipitate any debris that could clog the needle. 1µl of this solution was then loaded into a glass needle

(Eppendorf femtotips) by using microloader tips (Eppendorf) and injected into the posterior end of the aligned embryos. After injection embryos were allowed to hatch in a petri dish containing dH_2O at 27°C at ~80% humidity.

2.17. **Monitoring fluorescence in mosquito larvae**

Due to the nature of the embryo injection transient EGFP expression was mainly observed in the abdomen of the injected L1 using a 10x objective of a Nikon TE200 inverted microscope. The filters used for detection had an excitation at 439nm (CFP) or 484nm (EGFP) and emission of blue light at 476nm or green light at 507nm. Germline integration occurred in the G_0 parent if some of the following G_1 generation showed 3xP3 dependent EGFP expression in the eye and ganglia. From stably transformed G_1 individuals, homozygous lines were produced.

2.18. **Rearing of transient fluorescent mosquitoes**

After injection, males and females that had shown transient EGFP expression as larvae were separated at pupae stage before mating with adult wild type females and males at a ratio of 1: 10 respectively. Survivors which did not show any transient expression of GFP were crossed with each other. All mosquitoes underwent up to 3 reproduction cycles by repeated blood meals to increase the chance of finding transgenic offspring.

2.19. **RNA extraction**

1-5 mosquitoes were cold anaesthetised and homogenised with a homogeniser in Tri-reagent (Helena BioSciences Ltd.) containing phenol and guanidine thiocyanate that inhibit rapidly cellular RNase. By adding chloroform after 5min the homogenate was separated into aqueous phase containing the RNA, the interphase with the DNA and the organic phase containing the proteins. The followed centrifugation step (10000g for 10min at 4°C) was performed to pellet cellular debris and to recover the RNA

containing supernatant. Next RNA was precipitated with 0.7 volumes of isopropanol and finally washed with 70% ethanol and solubilised in 20µl RNase free dH2O.

2.20. **Reverse Transcriptase PCR (RT-PCR) to detect polyQ transcripts**

RT-PCR was used to amplify partial httex1polyQ transcripts. In a reaction volume of 20µl containing 10µl Reaction buffer (Promega) 2µg of total RNA was treated with 2 units DNAse1 (Promega) for 60min at 37°C to remove any genomic DNA that could be amplified by PCR. The reaction was terminated by adding 2µl DNAse stop solution containing guanidinium thiocyanate and heated at 65°C for 10min. This was followed by the cDNA synthesis. Firstly, 2µl Oligo dt (0.5µg/µl) and 2µl dNTPs (10mM of CTP, GTP, ATP and TTP) were incubated with the reaction at 65°C for 5min to denature the RNA templates and quickly stored on ice. After centrifugation the samples were divided into 2 aliquots. After adding 5x First-Strand Synthesis buffer, 2µl 0.1M DTT and 1µl Super RNA inhibitor solution the mixture was kept in a water bath at 42°C for 2min to allow the binding of the oligo dt's to the RNA. Then 1µl Superscript II reverse transcriptase (50 units) was added to one of the tubes only (+RT), while the other tube served as a control (-RT) and incubated at 42°C for 50min. Finally, the cDNA synthesis was terminated by heating to 70°C for 15 min. 1 unit Rnase H was added to each reaction to remove remaining RNA, incubated at 37°C for 20 min and stored at -20 degrees. For the PCR reaction 2µl (1/10 volume) of the cDNA (+RT and –RT) was then used as templates for a standard PCR.

Table 2.2 Primers used to amplify polyQ and the endogenuous S7 transcripts

Primer name	Primer sequence 5'-3'	Application	Expected amplicon size
PolyQRT_fwd PolyQRT_fwd	CCCTGGAAAAGCTGATGAAGG CGGCTGAGGCAGCAGCGGCT	amplification of htt exon1	httex1p20Q-185bp httex1p52Q-281bp httex1p69Q-332bp
S7RT_fwd S7RT_rev	GGCGATCATCATCTACGTGC GTAGCTGCTGCAAACTTCGG	amplification of the enogenuous S7 transcript	460bp

2.21. **DsRNA synthesis**

DsRNA was generated for RNA interference with *An. gambiae* specific transcripts. Firstly, the target regions were amplified by standard PCR from *An. gambiae* cDNA using primers with flanking *T7* promoter sequence (5'-TAATACGACTCACTA TAGGG-3') and inserted into the T-easy vector (Promega). This was followed by PCR using the same primers. 0.6µg of the PCR product was then used for the transcription reaction using the *T7* megascript kit according to the manufacturers instruction (Ambion). The resulting dsRNA was purified using MegaClear (Ambion) and suspended in 20ul dH_2O. RNA concentration and purity was determined with a Nanodrop Spectrophotometer at 260 and 280nm. Thereby at 260nm an OD of 1 unit corresponds to ~50µg/ml of RNA. The purity of the sample is represented by the 260/280nm ratio (high purity ~1.8). Further the presence of one single RNA band was confirmed by gel electrophoresis.

Table 2.3: Primers used for dsRNA synthesis. Additionally each of the primers contained a *T7* primer sequence at the 5' end (not shown).

Gene ID	Primer 5' -3'	Amplicon size
AGAP005712 (*PAH*)	GTCTGCCTGATCTTCTCG	309bp
	GGCTTCGTTATCCTTGTAGTC	
AGAP004978 (*PPO9*)	ATGTTTCCCACACGGTTC	501bp
	TCTTCCCAGCGATGATTCAAG	
AGAP006023 (*TH*)	CTTCGCCCAGTTCTCGCAA	529bp
	GAGTGAGACGGAAGGGTGTT	
AGAP009091 (*DDC*)	CGAGCTGGAGGTGGTTATG	572bp
	TGTGCGGGTTGAAGTTGAAC	
LacZ	AGAATCCGACGGGTTGTTACT	550bp
	CACCACGCTCATCGATAATTT	

2.22. **Adult mosquito injections**

DsRNA was diluted with dH_2O to a concentration of 1.5µg/µl. One day after adult emergence 69ul of this solution was injected into the thorax of CO_2 anesthetised females as described previously using a Nanoinject II injector (Drummond scientific) (Blandin et al., 2002). 3-4 days after injection these mosquitoes were blood-fed. One day after the blood-meal 3 mosquitoes were quick frozen and stored at -80C in Tri reagent (Helena Biosciences Ltd.) until their RNA was extracted transcribed to cDNA and analysed using qRT-PCR.

2.23. **qRT- PCR**

In order to examine the tissue specific expression and gene knockdown in mosquitoes a quantitative real-time PCR (qRT-PCR) was performed. 1-6 independent biological replicates (containing 3 mosquitoes each) were subjected to duplicate technical assays. Primers were designed to overlap exon-exon junctions. Each PCR reaction contained 300nM forward and reverse primer, 7.5µl FAST SYBR Green Mix (Applied Biosystems), 5µl of 10 fold diluted cDNA and was carried out in a Applied

Biosystems Prism 7500 thermocycler using the following program: 95 °C for 15 min, then 40 cycles (95 °C for 15 s, 60 °C for 60 s) followed by a dissociation curve. Results were only analyzed if the dissociation curves for each gene showed a single peak, and there was no amplification in the no-template controls. Analysis was performed by using the ΔΔCt method of relative quantification. This method required an approximately equal amplification efficiency of the target gene and endogeneuous gene. The ribosomal protein of RpL19 (AGAP004422), which is expressed at similar levels in different tissues, was used for normalization of the cDNA templates and to calculate threshold values (Rogers et al., 2008). Below is a list of genes that were analysed using qRT-PCR.

Table 2.4: Primers used for qRT PCR analysis

Gene ID	Primer 5' -3'	Amplicon size
AGAP005712 (*PAH*)	GGATGAGTTTGTGGAGAAGC	142bp
	CTTGTCGGTCAGGCAGTA	
AGAP004978 (*PPO9*)	TTCTGATTACAAGGACGATGCTG	84bp
	CGTAGACCACAGTATGAATGTGA	
AGAP006023 (*TH*)	AAGGAGATTGCCGAGATTGC	124bp
	GCACCATCAGCTCCTTCAC	
AGAP009091 (*DDC*)	CGAAGGAGATGGTCGATTACA	144bp
	CCATCACCTCTTCCCACTTT and	
	CGAAGGAGATGGTCGATTACA	73bp
	TGAACGGTCGGCAGAACA	
AGAP004422 (*RPL19*)	CCAACTCGCGACAAACA	61bp
	ACCGGCTTCTTGATGATCAGA	

2.24. **5' RACE**

The rapid amplification of cDNA ends (5'-RACE) is a polymerase chain reaction-based technique developed to facilitate the cloning of sequences from the 5' ends of mRNAs (Sambrook and Russell, 2001). According to the FirstChoice RLM-RACE

protocol (Ambion), 1µg total mosquito RNA was treated with calf intestinal phosphatise (CIP) to remove the 5' phosphate from molecules containing free 5' phosphates (ribosomal RNA, fragmented mRNA, tRNA and contaminating genomic DNA) leaving mRNAs unaffected. The RNA was then treated with tobacco acid pyrophosphatase (TAP) to remove the cap structure from the full-length mRNA leaving 5'- monophosphate. After the provided 5' RACE RNA adapter was ligated to the mRNA, a random-primed reverse transcription and nested PCR were performed to amplify the 5' end of the dopa decraboxylase (*ddc*) transcript. This reaction involved two sets of primers (outer and inner primer binding the 5'RACE adapter sequence: 5'-GCTGATGGCGATGAATGAACACTG-3' and 5'-CGCGGATCCGAACACTGCGT TTGCTGGCTTTGATG-3', the two antisense ddc specific primers: the outer primer CTCCTTCGCAAAGTCCTT-3' and inner primer ATGGATCCACTCTGGCGCCT GCATTT-3'), used in two successive runs of the PCR, the second amplified a secondary target within the first run product.

2.25. **Southern blot analysis**

Southern blotting was performed in order to verify the single site-specific integration and polyQ size of the httex1pQ constructs. For each transgenic line 10µg of DNA was digested with *HindIII* which flanks each of the polyQ region, the GFP marker and CFP region of the *attP* line E. Digested DNA was then separated on a 0.8% agarose gel, depurinated by exposure to UV (312nm for 2min) and washed with alkaline denaturation buffer (0.5M NaOH, 1MNaCl) twice for 20min. By means of capillary action DNA was transferred for 6-8h onto a Hybond-N+ charged nylon membrane (Amersham). Afterwards the membrane was neutralised with 3xSSC (20xSSC: 3MNaCl, 0.3M sodium acetate) for 5min and crosslinked under UV light for 1min (1500J/cm^3). The membrane was then incubated in prehybridisation buffer (100ml: 50ml 0.5M Na2HPO4 [pH 7.2], 35ml 20%SDS, 200ul 0.5M EDTA, 10ml 10% BSA) under constant rotation at 55ºC for 2h. Afterwards the prehybridisation buffer was replaced with fresh buffer containing 50µl of the denatured [α-^{32}P] ATP probe and hybridised in a rotator overnight at 55ºC. This probe was prepared according to the protocol of the High Prime DNA Labelling Kit (Roche Diagnostics). Primers flanking the *SV40* terminators of the httex1pQ construct and the *attP* insertion site were used to

amplify a 309bp DNA template (*SV40*- fwd 5' GCTCTAGA TAGATCATAATCAG CCATACC-3' and *SV40*- rev 5'-GGAATTCCATATGAAGCGATGAGATCTGCT AGC-3'). After gel extraction 25ng of the DNA (1-2µl vol, max 10µl) was denatured at ~100ºC for 10min and then immediately stored on ice. Then on ice 4µl High Prime Labelling mixture (1U/µl Klenow polymerase, 5x stabilised reaction buffer) ,2µl of unlabelled dCTP,dGTP, dTTP (0.075mM each), 2µl [α-^{32}P] ATP (10 uCi/µl, 6000 Ci/mmol)) (Perkin Elmer) and water (to 20µl total vol) was added to the probe and the solution was incubated for 20min at 37ºC . The reaction was then terminated by 2µl EDTA pH 8.0 and 28µl STE buffer (10mM Tris-Cl [pH 8.0], 0.1mM NaCl, 1mM EDTA [pH 8.0] in a final volume of 50µl. Unincorporated dNTPs were removed by centrifugation at 500g for 1min in ProbeQuant micro Sepahdex G50 DNA columns (GE Healthcare). The remaining radioactive probe was added to the column resin and centrifuged at 500g for 2min. Afterwards the probe was denatured at 100ºC for 10min and transferred immediately on ice until it was added to the prehybridisation buffer.

After probe hybridisation, firstly the membrane was washed with 3xSSC, 0.5% and then with 1xSSC, 0.5% SDS. Each washing step was performed in the rotator for 15min at 50ºC. Probe visualisation was then performed using a FUJIFILM-FLA-5000 Phosphoimager (Fuji Photo Film Co. Ltd, Stamford, CT, USA).

2.26. **Western blot analysis**

In order to detect the httpolyQ proteins, whole mosquitoes or 10 mosquito heads of the *attP* line E (control), httex1p20Q, httex1p52Q and httex1p69Q -line were homogenised in 50µl a) 1xPBS and freeze thawed for 3 times b) RIPA buffer (50mM Tris HCL pH 7.4, 50mM NaCL, 0.1% SDS, 5% Na Deoxycholate, 1% Triton X-100) or c) a lysis buffer containing 1% Triton X-100, 150mM NaCL, 50mM Tris HCL, 1mM EDTA that had been used in *D. melanogaster* . Thereby 1x protease inhibitor mix (Roche Diagnostics) was added to all lysis buffers immediately before homogenisation. After incubation of 30min on ice the samples were centrifuged for 5min at 13000rpm. The pellet containing cell debris and insoluble proteins was resuspended in 50% RIPA buffer. Then 14µl of the supernatant or the pellet were mixed with 5µl LDS sample Loading buffer and 1µl NuPAGE Reducing agent (both invitrogen) and boiled at 100°C for 10min. In the NuPAGE Novex system

(Invitrogen) that contained 1x MES SDS running buffer the proteins of the samples were separated according to their molecular weight at 150V for 1h on a 4-12% discontinous Bis-Tris gel. 10µl of SeeBlue Plus2 (Invitrogen) was used as size marker. The proteins were then transferred at 30V for 1h to a nitrocellulose membrane in a XCell II TM module that contained 1x NuPage Transfer buffer, 0.1% NuPage Antioxidant solution (all from Invitrogen) and 10% Methanol (Sigma Aldrich). Non-specific binding of proteins on the membrane was blocked by incubation with 2% BSA and 5% milk in 1x TBST (150mM NaCl, 10mM TrisHCl and 0.1% Tween 20) for 1h at RT or at 4°C overnight on a shaker. The membrane was probed in a solution that contained 2% milk, 1% BSA and the mouse MAB1574 (1:5000, Chemicon) or the sheep S830 primary antibody (1:1000, provided by Gilian Bates, Kings College London) for 1h RT or overnight at 4°C on a shaker. After 4x washing with 1x TBST (1x15min, 3x5min) the membrane was incubated for 1h with 2% skimmed milk (Marvel), 1% BSA (Sigma Aldrich) and the secondary antibody anti-mouse HRP (1:10000), anti-mouse AP (1:7500) or anti-sheep HRP ab7111 (1:10000, Abcam) and subsequently washed again for 4 times.

The following detection depends on whether the secondary antibody was linked to the reporter enzyme alkaline phosphatase (AP) or horseradish peroxidase (HRP) (both Jackson Laboratories).

For the alkaline phosphatase reaction the membrane was stained in 15ml alkaline phosphatase buffer (0.1M Tris-CL pH9.5, 0.1 NaCl, 0.05 $MgCl_2$, 0.1% Tween (Sigma Aldrich) which contained 50mg/ml BCIP, 100mg/ml NBT (both Roche Diagnostics). When the desired intensity of signal was reached, the reaction was stopped with transfer to dH2O for 20min.

The horseradish peroxidase-linked secondary antibody is used in conjunction with a chemiluminescent agent (2.5ml of detergent 1 and 2, Amersham ECL- Western blottong Analysis system, GE Healthcare) that produced luminescence in proportion to the amount of protein. The detection of this reaction was performed under exposure of light with a photographic film (Amersham Hyperfilm ECL, GE Healthcare) or a luminescent Image analyzer (Fujifilm, LAS 3000).

2.27. **Nuclear and Cytoplasmatic extraction of proteins**

The extraction of nuclear proteins of homogenised mosquito heads was performed according to the Proteojet cytoplasmatic and nuclear protein extraction kit (Fermentas). After cell lysis the non-denaturated cytoplasmatic proteins were separated from the nuclei by centrifugation (500g for 7min at 4C). The intact nuclei were washed with nuclei washing buffer and lysed by nuclei lysis buffer. The nuclei and cytoplasmatic fraction was compared on a 4-12% Bis-Tris gel (Invitrogen) by staining with Coomassie Blue (Sigma Aldrich).

2.28. **GC-MS analysis**

To measure volatile compounds like phenylalanine and its metabolites within mosquito tissues Gas chromatography-mass spectrometry (GC-MS) was applied. 3-4 mosquitoes were extracted in ice cold 80% methanol. After centrifugation the supernatant was transferred to an Agilent vial (Agilent Technologies UK Ltd) containing retention time locking (RTL) and quantification standards (5µl of 3mg/ml myristic acid d27 solution (in water/Methanol/isopropanol, 2:5:2), 20µl 13C Glucose solution (water/MeOH 1:1), 20µl 2,3,3-d3- Leucine standard solution (water/MeOH, 1:1). Then the sample was dried thoroughly in a SpeedVac concentrator (Eppendorf). After adding 10ul of methoxyamine derivatizing solution (40mg/ml methoxyamine (in anhydrous pyridine) the sample was vortexed and incubated at 30ºC for 30min. After 90µl MSTFA derivatizing solution was added, the sample was vortexed and incubated for 30min at 37ºC. Lastly 20µl 2-Fluorobiphenyl solution (1mM in anhydrous pyridine) was added as injection standard to the sample. GC-MS data acquisition was performed with an Agilent 5975 GC/MS detector (Agilent Technologies UK Ltd.) by Volker Behrends from Imperial College who performed the subsequent peak identification and quantification of the extracted metabolites as well (Behrends et al., 2011). He developed a software complement to AMDIS which allowed the identification of additional peaks which were not recognised by AMDIS (due to shift in retention time).

For data analysis, the blank (contained all standards, but no mosquito tissue) was subtracted from the metabolite concentrations of the test samples and divided each metabolite of the test samples by metabolite of the respective control samples.

2.29. **Blood-feeding**

For general maintenance and experiments which involved oviposition or life span assays of dsRNA injected or transgenic mosquitoes, female *An. gambiae* mosquitoes were blood-fed on anaesthetised mice for 20min. All animal work was conducted according to UK Home Office Regulations and approved under Home Office License PPL 70/6453.

In order to measure the survival and intake of mosquitoes of phenylalanine metabolites, phenylalanine or phenylpyruvate was added to human blood at a final concentration of 10, 25 and 50mM and fed to mosquitoes using a standard glass feeder (www.MR4.org). The system used contained an outer area with circulating warm water (37ºC) and an inner chamber into which the blood is poured. The bottom of this chamber was covered with thin parafilm which allowed the mosquitoes to pierce through and feed on the supplemented blood for 20min.

2.30. **Infection assay**

4 days after mosquitoes were injected with dsRNAi, ~140 mosquitoes (~30-40 per cup) were offered a *P. berghei* infected mouse with ~5-10% parasitemia (kindly provided by Tibebu Habtewold, Imperial College London). The parasite strain expressed the green fluorescent protein (*GFP*) in the ookinete and oocyst stages, which permits convenient and quantitative monitoring of the infection (Vlachou et al., 2004). Because *P. berghei* does not develop below a temperature of 21ºC, blood-fed mosquitoes were kept at 19ºC until they were dissected 8 days after infection (Dimopoulus et al., 1998). For the dissection, the mosquito was anaesthetised with CO_2 and then using a forceps the last two segments were pulled revealing the midgut and ovaries. The midgut was then mounted on Vectashield (Vectorlabs) and investigated for GFP and melanised oocysts using a fluorescence/bright field

microscope a 10x objective of a Nikon TE200 inverted microscope (**Appendix Figure 10.4**).

2.31. **Turning response assay**

The turning response assay was modified from Bellen et al. studying the flight response of fly mutants after CO_2 exposure (Bellen et al., 1992). A group of 13 one day old mosquitoes were anaesthetised on a CO_2 pad for 15sec causing mosquitoes to lie on their back. After 2.5min the number of mosquitoes able to raise themself was recorded. The procedure was repeated 3, 9, 14 and 24 days after adult emergence using the same mosquitoes.

2.32. **Oviposition assay**

10-20 female mosquitoes were allowed to mate for 4 days before they were given a blood meal and placed into single plastic cups aligned with 5cm filter paper strip and filled with 50ml larval rearing water (Thailayil et al., 2011). In case of the oviposition assay with dsPAH injected mosquitoes, mating couples were collected and then injected with dsPAH 24h after mating. Mating was induced by placing ~30 3 day old females into a cage with ~ 200 4 day old male mosquitoes. Couples were collected during copulation in modified plastic falcon tubes (covered by net) as described previously (Thailayil et al., 2011). 3 days after injection the mosquitoes were blood-fed. The numbers of eggs laid and larval hatching rate was recorded and statistically analysed using the Mann-Whitney test (comparing number of eggs) or by t-test of the arcsine transformed proportion of hatched eggs.

2.33. **Chromatic vision assay**

Mosquitoes prefer oviposition in black filter paper coverd egg bowls over white filter paper canopied oviposition bowls (snowKennedy, 1942). In another experiment Snow found that illuminated oviposition sites by light of 610 m/i and all longer wavelengths (red) elicited the highest response (52%), and the 470-610-m,u (green) site the lowest

(12%), with an intermediate number (36%) of eggs being deposited at the 360-500-m// (blue) site (Snow, 1971).

In order to establish whether the visual behaviour of dsRNA injected and polyQ expressing mosquitoes is affected, ~10-20 gravid females were allowed to choose from 4 different colur covered egg bowls (white, red, yellow and black paper,) placed in each in a corner of a 25x25cm wide cage. The number of eggs laid into each egg bowl was a preference indicator and reflected the ability to differentiate between the different colours. No colour vision was expected to result in an equal number of eggs laid per egg bowl.

2.34. **Fly strains**

The generated plasmids httN-25QEGFP and httN-97QEGFP were injected into the fly line *y1 w67c23; P{CaryP}attP2* (Bloomington stock 8622) by Sang Chan (Cambridge University). The used marker- less fly line contained an *attP* site that was created by P- element insertion chromosome 3L, band 681A–B2, between genes *CG6310* and *Mocs1* thus allowing site- specific *attB/attP* mediated integration of the attB carrying httN-QEGFP plasmids (Groth et al., 2004). The double balanced line w^{1118}*; TM2, Ubx/TM6C,Sb* which stabilises chromosome 3 and carried the homozygous lethal and heterozygous visible mutant *Sb* (stubble) and *Ubx* (ultrabithorax) gene as a marker was used to then generate homozygous httNQEGFP flies (Ryder et al., 2004). Whilst flies with an *Ubx* mutation have reduced halters the *Sb* mutation results in shortened, stubbly hairs on the back of the fly, which is easily visible under a microscope and was therefore chosen for selection in this experiment.

2.35. **Fly rearing**

All *D. melanogaster* lines were reared in fly vials containing fly food (6ml water, 69mg agar, 0.6g dextrose, 0.6g maize, 0.1g yeast, 0.2ml Nipagen) at 18-20°C (Sang Chan, Cambridge University). For maintenance flies were transferred to a new vial containing fresh food every 2-3 weeks.

2.36. **Embryo microinjection in flies**

All fly embryo injections were performed in cooperation with Sang Chan (Cambridge University). HttN25QEGFP and httN97QEGFP plasmids were injected with the Integrase RNA into eggs of the fly line *y1 w67c23; P{CaryP} attP2* (Bloomington stock 8622) according to the protocol of Fish (Fish et al., 2007). The surviving injected adults were crossed with the white eyed w^{1118} line at a ratio of 1:4. *attp* fly. Subsequently all virgin G_1 httN-QEGFP flies were crossed individually in a ratio of 1:4 to the double balanced line w^{1118}*; TM2, Ubx/TM6C,Sb.* The resulting 25% of the G_2 progeny which had visble GFP fluorescent eyes (httNQEGFP) and stubble bristles were then crossed with each other giving homozygous httN-QEGFP G_3 progeny with normal bristles.

2.37. **Monitoring fluorescence in adult flies**

EGFP expression was observed 3xP3 promoter-specific in the eye of transformed adult flies using a 10x objective of a Nikon TE200 inverted microscope at an excitation at 484nm and emission at 507nm.

2.38. **Climbing assay**

The climbing assay was peroformed to determine the locomotory behaviour of the 25 and 97 polyglutamine expressing flies. 15 flies were tapped to the bottom and after 45sec the number of flies (n) on the top and bottom of the vial was determined. For each vial this procedure was repeated 3 times. The average was used to calculate the performance index (PI) = 0.5*(n total +n top -n bottom)/ n total) (Rival et al., 2004). At each time point the PI of the control was compared to the test group using a student's t-test.

2.39. **DNA extraction from mosquito/flies**

The DNA of 1-10 adult mosquitoes/flies was extracted using the Promega Wizard genomic DNA Purification Kit. The cells and Nuclei were lysed in chilled Nuclei

Lysis solution by using a motorised tissue grinder (VWR) and incubated for 15-30min at 65°C. RNA and proteins were removed subsequently by incubation with RNase A and protein precipitation solution followed by centrifugation. The supernatant containing the DNA was then precipitated with isopropanol, washed with 70% ethanol and rehydrated in rehydration solution (10mM Tris-HCL, 1mM EDTA) at 65°C for 1h. It was then stored at 2-8°C until further analysis.

2.40. **PCR of genomic DNA**

The PCR was assembled as with plasmid DNA using 25ng genomic DNA and TAQ or Phusion Hot Star for amplification. The latter enzyme was used with the following program: 98°C for 30 s, 30-35 cycles of 98°C for 1min, 55-70°C 30s, 72°C 30s-1min and 72°C for 10min. The primers used for genomic PCR reactions are shown in **Table 2.5.**

Table 2.5: Primers used to amplify genomic DNA

Primer 5'-3'	application	Amplicon size
CTCATGTAACAGTTCATAGTTCTCGC ATCGCTGAGATAGGTGCCTCACTGA	site-specific integration of httex1p20Q, httex1p52Q and htt ex1p69Q into the *attP* line E	2264bp
CAGGTCAGAAGCGGTTTTCGG GCGGCTCGAGGGT ACCTCTAG	site-specific integration of httex1p20Q, httex1p52Q and htt ex1p69Q into the *attP* line E	1051bp
CCCTGGAAAAGCTGATGAAGG GGTGCAGCGGCTCCTCAGC	determination of the polyQ length of the httex1pQ lines	expected: httex1p20Q-249bp httex1p52Q-345bp httex1p69-396bp
GATCCGCATGGCGACCCTG CTAGAGGTACCCTCGAGCCGC	determination of the polyQ length of the httex1pQ lines	expected: httex1p20Q- 309bp httex1p52Q- 405bp httex1p69- 456bp
ATCGCTGAGATAGGTGCCTCACTGA CTTGTGTCATGTCGGCGAC	site-specific integration of httNQEGFP into *y1 w67c23; P{CaryP}attP2*	1315bp
ACTGCAACCCATTCACACTG ATCGCTGAGATAGGTGCCTCACTGA	site-specific integration of httNQEGFP into the X1 insertion line	1981bp
CCCTGGAAAAGCTGATGAAGG TCGTGCTGCTTCATGTGG	determination of the polyQ length of the httNQEGFP fly and mosquito lines	httN25QEGFP-523bp httN97QEGFP-723bp

2.41. **Immunostaining and Confocal microscopy**

All fly dissections and subsequent immunostaining procedures were performed at the National Institute for Medical Research (NIMR) with help of Holger Apitz. Fly brains were dissected in ice cold phosphate buffered saline (PBS) and fixed by incubation in 2% paraformaldehyde in 0.01 M L-lysine monohydrochloride (Sigma Aldrich) and

0.05 M sodium phosphate buffer, pH 7.4, for 1h. This was followed by rinsing in 0.5% Triton X-100/PBS (PBT). In order to prevent unspecific binding the brains were treated with 10% normal goat serum (NGS) (Jackson ImmunoResearch) in PBT before they were incubated with primary antibody for 6-12h at 4ºC. Antibodies used were mAb24B10 (anti-chaoptin; 1:75; Developmental Studies Hybridoma Bank) and PKC zeta (C-20): sc-216 (Santa Cruz Biotech). After rinsing in PBT, secondary antibodies labelled with Cy3 and Cy5 (Jackson ImmunoResearch) were applied in 1:200 dilution for 2.5h and then washed 3 times in PBT. The preparations were mounted in Vectashield and confocal images were collected by Holger Apitz with a Leica TCS SP5 confocal microscope equipped with a resonant scanner using a 20× (0.7 numerical aperture (NA)) air objective or 40× (1.25 NA) and 100× (1.46 NA) oil objectives. Stacks of images were acquired using a 488-nm argon laser line for EGFP (acousto-optical beam splitter (AOBS) setting, 490–515 nm), a 561-nm laser for Cy3 (AOBS settings, 572–639 nm), and a 633-nm laser for Cy5 (AOBS settings, 650–711 nm). Images were analysed by Holger Apitz using Volocity (Improvision PE) and ImageJ (NIH) software (Collins, 2007).

Subsequent dissections and immunostaining of mosquito brains were conducted at Imperial College London whereby the fly protocol was slightly modified: Because the *An. gambiae* brain tissue was found to be more fragile, the whole head was fixed and then dissected prior immunostaining. Primary antibodies used were nc82 (anti-Brp, bruchpilot; 1:50; Developmental Studies Hybridoma Bank), mAb24B10 (1:50, Developmental Studies Hybridoma Bank) and ELAV-9F8A9-s (1:10; Developmental Studies Hybridoma Bank). The secondary antibodies were anti-rat Alexa 568 (1:200; Invitrogen) and anti-mouse Alexa 647 (1:200; Invitrogen). ImageJ was used for analysis.

2.42. **Life span assays**

All *An. gambiae* mosquitoes and *D. melanogaster* flies were reared under the conditions described under 2.15. In general, for the survival analysis, at each day a death occurred, survival probabilities (S(*t*) = number of individuals still alive at time *t* / total number of individuals in the study) and survival rate (= product of the survival probabilities) was estimated using the Kaplan-Meier method (SPSS). Non-natural

deaths mosquitoes sticking in the glucose or cage were censored i.e. such mosquitoes were only included in the calculation of the survival function prior to their death. The median survival time was calculated as the survival time for which 50% of the mosquito population had died. Some experiments were terminated prior to this point, in which case their median survival time was not calculated. A survival curve was obtained by plotting the proportion of surviving mosquitoes (or %) at the different time points until the end of the observation. The obtained curves of different groups (different dsRNA injections or transgenic constructs) were then analysed using the log-rank test. A p-value of < 0.05 was defined to be significant.

The specific life span assays were performed as follows:

In the first phenylalanine/phenylpyruvate assay ~70 mosquitoes were fed either with glucose alone (control) or additionally with 10, 25, 50 or 100mM phenylalanine or phenylpyruvate over a period of 7 days. Each day the number of dead mosquitoes was counted. In the second phenylalanine/phenylpyruvate assay, ~20 mosquitoes fed on a blood-filled membrane supplemented with 0, 10mM, 25mM or 50mM phenylalanine or phenylpyruvate and the number of dead mosquitoes was recorded daily until 7 days after blood meal. In order to determine in these 2 phenylalanine/phenylpyruvate assays whether the concentration of phenylalanine or phenylpyruvate had an effect on the mosquito survival a Cox regression (proportional hazard regression) was performed in SPSS (Cox, 1972). Briefly, the procedure models or regresses the survival times (or more specifically, the so-called hazard function) on the explanatory variable in this case concentration of supplemented phenylalanine or phenylpyruvate.

In order to study the survival of dsPAH, dsPPO9, dsTH and dsLacZ injected mosquitoes, only mosquitoes that were blood fed were transferred to paper cups with access to 10% glucose (~8cm diameter, 10cm height) (~30-40 mosquitoes per cup). For this all mosquitoes were CO_2 anaesthetised and examined whether they have had blood-filled abdomen.

To measure the survival of transgenic htt expressing female and male mosquitoes 25-50 unmated individuals were reared in 25cm x25cm small cages with access to 10% glucose. Every 3-4 days dead mosquitoes were removed.

The survival of 15-60 httN25QEGFP and httN97QEGFP expressing female and male *D. melanogaster* was measured in food vials (diameter 3cm) (15 flies per vial). Every 4-5 days the surviving flies were transferred to new vials containing fresh food.

3. Chapter: Manipulation of the phenylalanine/tyrosine metabolism in *An. gambiae* - Results

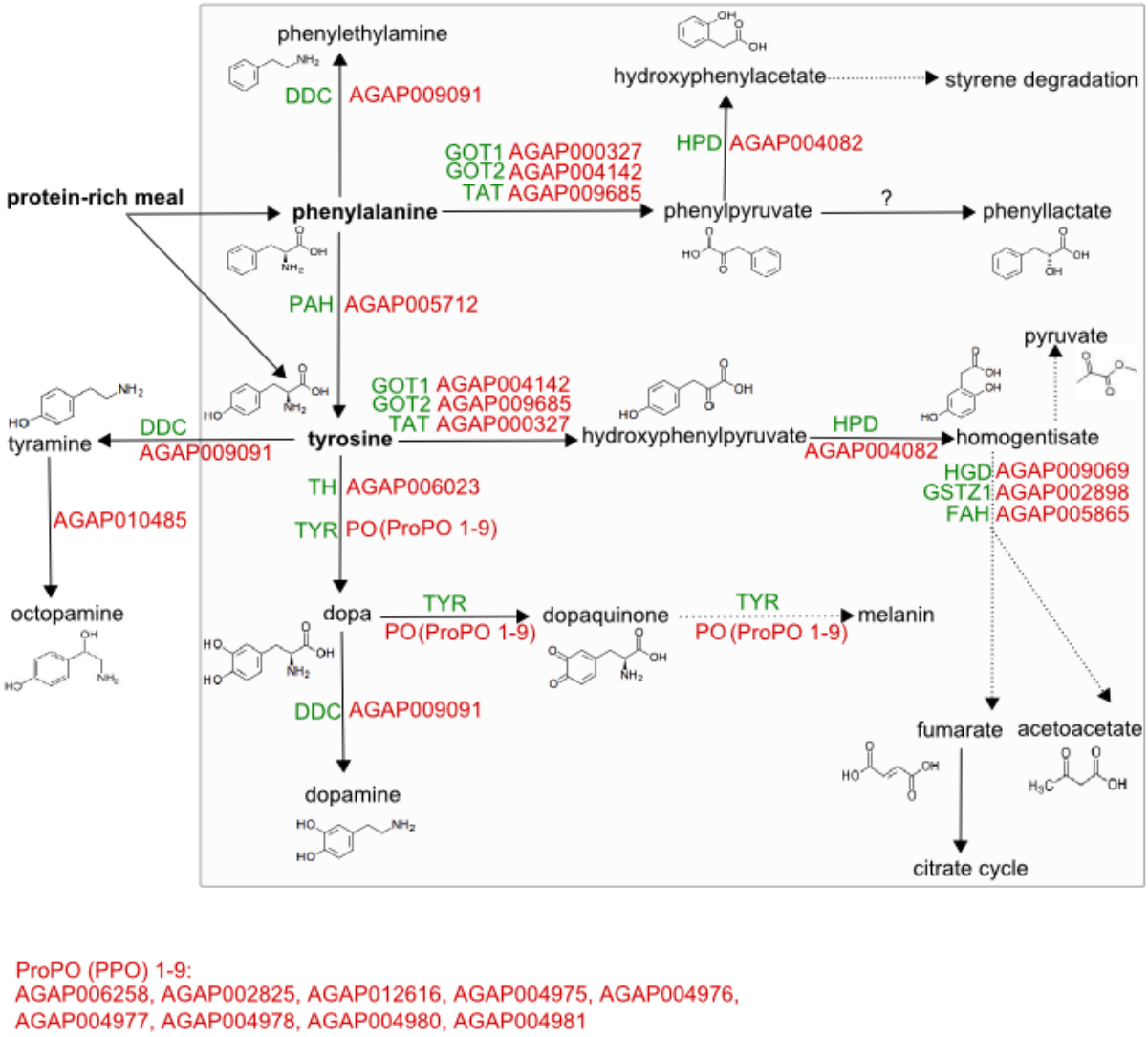
3.1. Identification of *An. gambiae* enzymes involved in phenylalanine metabolism

One approach to induce mortality in adult females is by formation of toxic metabolites in response to blood meal. In humans several metabolic diseases with malfunctioning enzymes involved in the phenylalanine pathway are known. One of them is phenylketonuria which is caused by a lack of the rate-limiting enzyme in the phenylalanine catabolism, phenylalanine hydroxylase (PAH) which leads to excessive phenylalanine and toxic transaminase products (phenylpyruvate and phenyllactate) that interfere with the energy metabolism and cause mental retardation (Krause et al., 1985).

A combined analysis of the Homologene database from NCBI and the Kyoto encyclopaedia of genes and genomes (KEGG) revealed homologous genes between humans and *An. gambiae* mosquitoes for the majority of enzymatic steps involved in the catabolism of phenylalanine or tyrosine which lead to the formation of glucose, fatty acid precursors as well as melanin and neurohormones (www.**ncbi**.nlm.nih.gov; http://www.genome.jp/kegg-bin/show_pathway?aga00360). Merely adrenaline and noradrenaline are unique to members of the deuterostome lineage (including humans) and are replaced functionally in *An. gambiae* by their invertebrate counterparts, the monoamines octopamine and tyramine (Roeder, 2005). **Figure 3.1** shows a summary of the identified *An. gambiae* and *H. sapiens* genes involved in the phenylalanine metabolism.The high conservation of the genes involved in this pathway implied a similar functional role and suggested that a similar disease phenotype could be induced by interfering with key enzymes.

In this project, one objective was to induce a Phenylketonuria (PKU) -like disease in mosquitoes. Because *An. gambiae* has homologous enzymes responsible for the hydroxylation of phenylalanine (AGAP005712) and alternative transamination of phenylalanine to phenylpyruvate, phenylethylamine and hydroxyphenylacetate (AGAP009091, AGAP004142, AGAP009685, AGAP000327 and AGAP004082) (all identified by KEGG and NCBI) the effect of a knockdown of the *An. gambiae* phenylalanine

hydroxylase was investigated to determine whether the same toxic phenylalanine metabolites as in PKU patients are formed.



ProPO (PPO) 1-9:
AGAP006258, AGAP002825, AGAP012616, AGAP004975, AGAP004976,
AGAP004977, AGAP004978, AGAP004980, AGAP004981

Fig 3.1: The phenylalanine/ tyrosine pathway is highly conserved between *H. sapiens* and *An. gambiae*. The rectangle shows the conserved part of the pathway between *H.sapiens* and *An. gambiae* generated by means of the Kyoto Encyclopaedia of genes and genomes (KEGG), the Homologene system (NCBI) and Roeder, 2005 (Roeder, 2005)). All metabolites are displayed in small letters; all enzymes are shown in either green (*H. sapiens*) or red (*An. gambiae* homologue). The following enzymes were identified: DDC,AGAP09091- dopa decarboxylase; GOT1 and GOT2, AGAP004142 and AGAP009685- aspartate aminotranferase; TAT, AGAP000327- tyrosine aminotransferase; HPD, AGAP004082- 4-hydroxyphenylpyruvate dioxygenase; PAH, AGAP005712- phenylalanine hydroxylase; TH, AGAP006023- tyrosine hydroxylase; HGD, AGAP009069- homogentisate 1,2 dioxygenase; GSTZ1, AGAP002898- glutathione transferase zeta; FAH, AGAP005865- fumarylacetoacetase; AGAP010485- dopamine beta- monooxygenase (homologous enzyme DBH converts dopamine to noradrenaline in humans).

3.2. **Phenylpyruvate is toxic for mosquitoes**

In light of investigating whether phenylpyruvate (PPA) the known effector of toxicity in PKU is toxic and decreases the life span of mosquitoes, 8 groups of ~70 female *An. gambiae* mosquitoes were fed a 10% glucose solution which contained ascending concentrations of either phenylpyruvate or phenylalanine (Phe) over a period of 7 days. The concentrations were referred from previous experiments to induce PKU in rats and mice (Agrawal et al., 1970, Gimenez et al., 1977).

A continuous decline in survival was observed in response of feeding mosquitoes with ascending concentratrion of PPA (Cox regression $p<0.001$). This trend was not observed for mosquitoes fed with ascending concentration of phenylalanine (Phe) or glucose only (control) (both Cox regression $p>0.05$) (**Figure 3.2 A**). This result is in accordance with other studies suggesting phenylpyruvate as main toxic agent in mammalian phenylketonuria models (Patel and Tilghman, 1973, Patel, 1972, Agrawal et al., 1970, Gazit et al., 2003a).

Because adult mosquitoes ingest their aminoacids/proteins needed for egg production from blood meal, in order to simulate a more realistic "Phenylketonuria" phenotype, mosquitoes were fed with a single blood meal containing ascending concentrations of phenylalanine and phenylpyruvate. While there was no effect on the survival at a low concentration of 10-25mM PPA a strong decrease in survival was observed at a concentration of 50mM of PPA (**Figure 3.2 B**). The survival of these mosquitoes decreased rapidly to 55% during the first 3 days after the blood meal. Comparing the mortality inducing effect of 50mM PPA between 10% glucose and blood fed mosquitoes, the decrease in survival occurred faster in blood fed mosquitoes. It is possible that the difference between blood and glucose 50mM PPA fed mosquitoes was due to a protective effect by the added glucose, which have been shown to able to reverse the toxic phenotype in rats after PPA treatment (Gazit et al., 2003b).

In order to determine whether this toxicity in response of feeding mosquitoes with ascending concentration of phenylalanine metabolites was due to PPA accumulation characteristic in urine and blood of PKU patients, a GC-MS analysis was performed. This technique had been used to identify biomarkers of human diseases and was applied here for the first time to examine the metabolome of whole mosquitoes. Overall, a total of 45 metabolites including phenylalanine metabolites were identified (**Appendix Table 10.2**). The comparison between phenylalanine fed mosquitoes and the control (10% glucose solution only) showed that the

phenylalanine concentration was higher in phenylalanine- fed mosquitoes (**Figure 3.3A**). This titre increased with ascending administered phenylalanine concentration.

However, the phenylalanine concentration did not increase with the time of administration of a period of 7 days. Since the glucose solution supplemented with phenylalanine was exchanged every day, a gradual degradation of the phenylalanine solution was excluded as a possible cause for this result. It seems more likely that this was caused by metabolisation of phenylalanine to other compounds downstream of the phenylalanine/tyrosine pathway. However, the concentration of phenylalanine metabolites such as phenylpyruvate (PPA), phenyllactate (PLA) as well as tyrosine (Tyr) did not correlate with the changing phenylalanine amount.

Samples containing extracted mosquitoes treated with 10-50mM PPA, showed a rise of the phenylalanine metabolite PLA which correlated positively with the administered concentration (**Figure 3.3 B**). This suggests that in mosquitoes phenylpyruvate is converted to phenyllactate. This phenylalanine metabolite has been associated with PKU and is increasingly found in the urine of "pheylketonuria" affected patients.

Another alternative hypothesis is that phenyllactate was produced by microorganisms, however, phenyllactate has been shown to have a strong antimicrobial activity against gram- and gram+ bacteria and so far only a few microorganisms are known to be able to produce phenyllactate from phenylpyruvate including *Geotrichum candidum* (Dieuleveux et al. 1998a), propionibacteria (Thierry and Maillard 2002) and lactic acid bacteria (LAB) (Lavermicocca et al. 2000; Strom et al. 2002), which naturally do not occur in the mosquito gut, blood or glucose food source.

With continuous administration, the PLA titre was not found to be further elevated and it was assumed that PLA was either metabolised or excreted. The latter seems more likely because according to KEGG so far no enzyme has been described in humans or mosquitoes to be able metabolise phenyllactate, which could account for its accumulation and subsequent toxicity.

Overall, the tyrosine concentration showed a similar pattern which was independent of the supplemented phenylalanine and phenylpyruvate concentration, but differed with the days of administration. Most likely this was caused by a change in the tyrosine concentration in the reference (control) samples. It is unknown what caused this change, but it was not linked to the total metabolite concentration of the different samples (**Figure 3.3**).

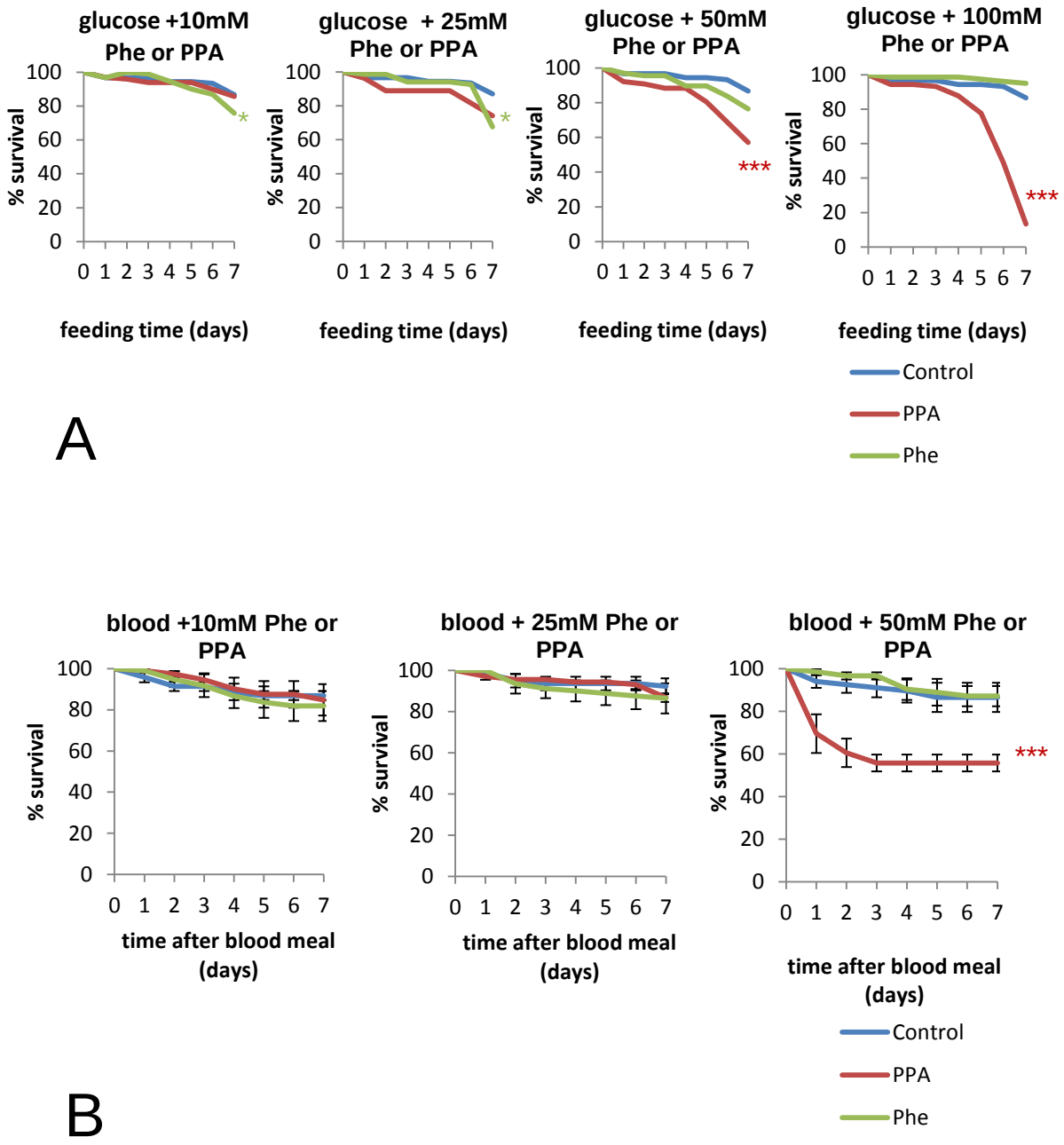


Fig. 3.2: Survival of female *An. gambiae* mosquitoes after ingesting glucose or blood supplemented with various concentrations of phenylalanine (Phe) or phenylpyruvate (PPA). A Survival of 54-90 mosquitoes fed with 10-100mM pheneylalanine or phenylpyruvate containing glucose. The control group was fed on glucose solution only. Log-rank test (phe or ppa vs. control): *p <0.05, ***p<0.001 **B** Survival of ~60 female *An. gambiae* mosquitoes that are fed on human blood containining 50mM phosphate buffer and 5mM ATP in the control or additionally 10-100mM phenylalanine (Phe) or phenylpyruvate (PPA). The graph shows the mean of 3 different repeats and the standard error of the mean. A log-rank test was performed (phe or ppa vs. control) for each independent repeat (ppa vs. control: 1. repeat p=0.06, 2. repeat p=0.02, 3. repeat p= 0.005; overall ***p<0.001.

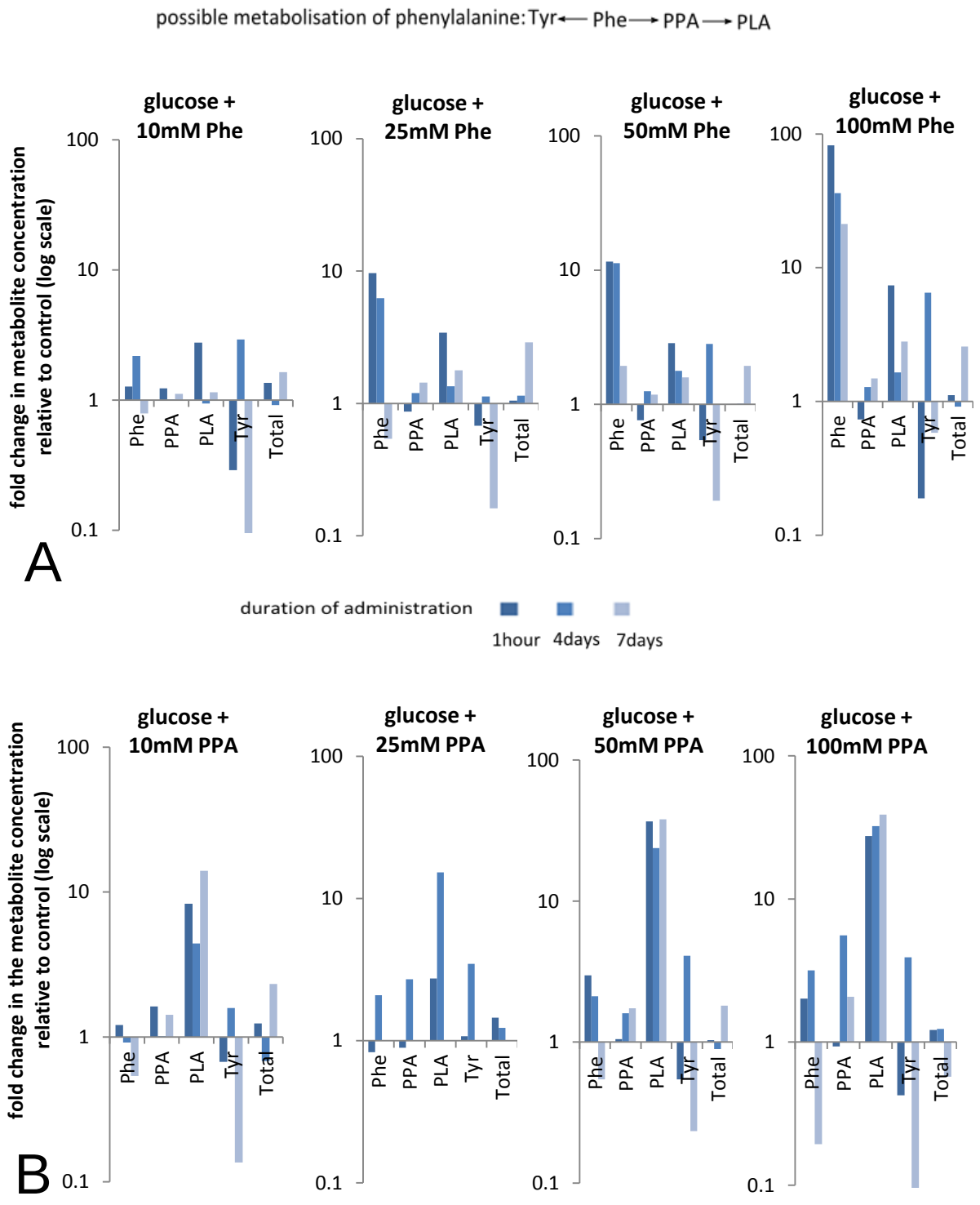


Fig. 3.3: GC-MS analysis of key amino acids in the phenylalanine metabolism in response to increasing concentrations of Phe and PPA. A Fold change of phenylalanine (Phe), phenylpyruvate (PPA), phenyllactate (PLA) and tyrosine (Tyr) in phenylalanine-fed mosquitoes relatively to the control (fed glucose only).“Total” refers to the relative total metabolite concentration found in the Phe or PPA administered mosquitoes compared to the control samples. Samples were extracted after 1hour, 4 and 7 days of continuous administration. **B** Fold change of Phe, PPA, PLA and Tyr in phenylpyruvate-fed mosquitoes relatively to the control (fed glucose only)**.** The sample containing the metabolited of mosquitoes fed on 25mM PPA over a perioid of 7 days is missing.

3.3. The phenylalanine metabolising enzyme PAH is increasingly expressed after a protein-rich blood meal

After it was shown that humans and mosquitoes share a conserved phenylalanine/tyrosine metabolism which metabolises toxic metabolites like phenylalanine pyruvate in a similar way, it was hypothesized that the putative orthologue enzyme phenylalanine hydroxylase (65% identity with human and fly PAH, **Appendix Figure 10.5**) plays an important role to metabolise phenylalanine in order to prevent the formation of phenylpyruvate.

Phenylalanine hydroxylase has been further shown in *Aedes aegypti* to facilitate the melanotic immune response and wound healing by providing the substrate for melanin synthesis necessary for an effective response against parasites (Johnson et al., 2003, Infanger et al., 2004). Therefore, disruptions in this pathway could cause potentially an accumulation of toxic phenylpyruvate or phenyllactate and effect on the immune response.

Firstly, it was examined whether PAH increases with the ingestion of a blood-meal containing protein-bound and free phenylalanine. Using vectorbase database a mRNA region common to the three differently spliced PAH transcripts in *Anopheles gambiae* (AGAP005712 RA, AGAP005712 RB and AGAP005712RC) was chosen for expression analysis (**Figure 3.4**). In a single experiment, PAH expression was increased in the head, carcass (including the fatbody), ovaries and midgut in response to a blood meal of 3 pooled mosquitoes (**Figure 3.5**). While, in the head and in the carcass tissue the peak expression was reached 3h post blood meal, PAH expression rose in the midgut until 24h post-blood meal and in the ovaries even until 48h post-blood meal. Since phenylalanine is the precursor of neurohormones (e.g. dopamine, octopamine and tyramine) as well as melanin which is needed for wound healing, immune response and egg shell tanning, possibly, the different expression pattern of PAH could reflect its various function specific to the expressing tissues. Further, the phenylalanine hydroxylase expression could be linked possibly to the location of hemocytes which have been shown to contain phenylalanine hydroxylase in *Aedes aegypti* and circulate the haemolymph or are attached tor abdominal organs such as midgut or trachea (Pinto et al., 2009).

48 hours post-blood meal the expression of PAH remained elevated in all tissues. Marinotti and collegues found a maintained high expression of PAH even 15 days post-blood meal (Marinotti et al., 2005). It is unclear why the PAH level remains at a high level throughout this time.

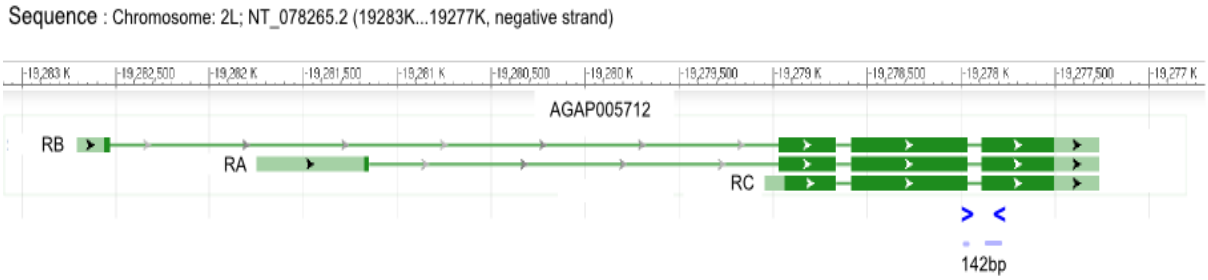


Fig. 3.4: Structure of the three different PAH transcripts of *Anopheles gambiae*. The dark green area displays the protein-coding region, the light green area the untranslated region (UTR) (Vectorbase database). Blue arrows indicate the primers spanning an intron region used for tissue specific amplification of PAH (resulting truncated PAH fragment: 142bp).

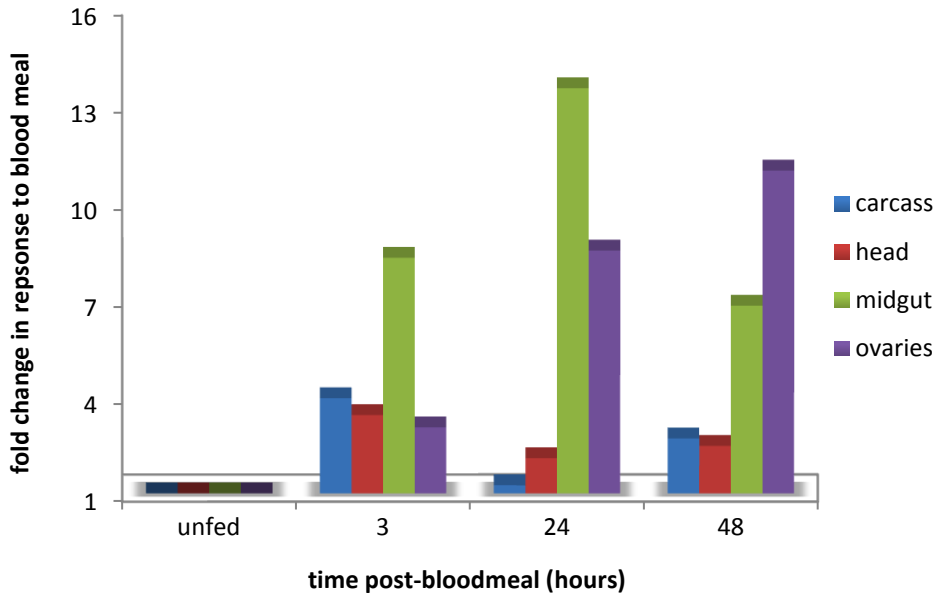


Fig. 3.5: Phenylalanine hydroxylase (AgPAH) expression in different female *Anopheles gambiae* mosquito tissues in response to a blood meal. The tissue-specific PAH expression at time 0 was used as reference. The endogeneous control was the ribosomal gene *RPL19*.

3.4. A knockdown of PAH does not reduce the life span in *An. gambiae* mosquitoes

Initially, the enzyme phenylalanine hydroxylase was targeted by feeding the toxic analogue to phenylalanine, chlorophenylalanine, which had been shown to reduce the liver PAH activity in mice to 10-20% of normal (Tong et al., 1972). However, when 100mM chlorophenylalanine was added to the blood it coagulated, probably due to the acidic nature of the chlorophenylalanine solution (pH 5.5). Subsequently, mosquitoes rejected this blood.

In order to avoid toxic effects that are unrelated to the disruption of the phenylalanine metabolism, it was decided that the knockdown of PAH using RNAi would be more suitable. RNAi is a post-transcriptional gene silencing mechanism through which targeted genes are silenced by introduction of double stranded RNA into the organism. In mosquitoes dsRNA introduction into adults has been most successful through injections into the thorax (Catteruccia and Levashina, 2009). Upon injection dsRNA interferes with the gene-specific transcript but not with the protein. Therefore, in order to determine the amount of existing protein prior RNAi and reduced protein synthesis after RNAi a Western blot analysis would need to be performed.

In six different experiments ~130 adult female mosquitoes were injected with dsPAH RNA and dsRNA against an unrelated bacterial gene *LacZ*, as a negative control. Three days after injection PAH transcript levels were assessed 24hours after blood meal by qRT PCR. Compared with the dsLacz controls, the analysis showed that the PAH expression was significantly reduced in dsPAH injected mosquitoes (average 33% knockdown, $p<0.05$) (**Figure 3.6A**). One reason for this relatively small knockdown could be that PAH was found not only in the fatbody but also in other tisses such as the ovaries **(Figure 3.5)** which can be refractory to RNAi, due to decreased uptake of dsRNA (Ciudad et al., 2007). Further, the type of gene can influence the RNAi results. Genes with efficient feedback mechanism of regulation might counteract depletion of mRNA with increased transcription leading to a fast recovery of mRNA levels (Belles, 2010, Ciudad et al., 2007).

In four independent experiments the remaining injected mosquitoes showed no significant difference in their survival between the control and dsPAH injected females (in each experiment $p>0.05$) (**Figure 3.6B**). In order to investigate whether multiple blood meals decrease the life span with reduced PAH activity, in another experiments 30 dsPAH and dsLacZ injected females were given 3 consecutive blood meals every 3 days. Again no

difference in survival was observed (**Figure 3.6C**). This absent phenotype implicated either that the blood meal contains insufficient phenylalanine to form toxic metabolites or that phenylalanine hydroxylase knockdown of 33% was too low which led to an insufficient accumulation of phenylpyruvate/phenyllactae in *An. gambiae* females.

In order to determine whether the knockdown of the phenylalanine hydroxylase had any physiological effect, it was hypothesised that a knockdown of the mosquito PAH would have led to change in the concentration of its substrate phenylalanine and its product tyrosine. In three different experiments three pooled mosquitoes were analysed using GC-MS analysis (**Figure 3.7**). Compared to the dsLacZ injected control, the body of dsPAH injected mosquitoes contained a significantly increased amount of phenylalanine (22-fold, paired t-test on log- transformed data, p=0.01) and phenyllactate (3fold, paired t- test on log-transformed data, p=0.01). The tyrosine level was on average 3 fold lower than dsLacZ control (3-fold, paired t- test on log- transformed data, p=0.07). This result suggested that in mosquitoes PAH is most likely the responsible enzyme to convert phenylalanine to tyrosine. Further, dsPAH RNA was able to knockdown the transcript and thus provide less PAH protein. Additionally, the GC-MS analysis showed that phenyllactate was formed, but at a relatively low concentration compared to the lethal ~30 fold increase found in 50mM and 100mM PPA fed females. This difference could possibly explain, why no increased mortality was observed in dsPAH injected mosquitoes.

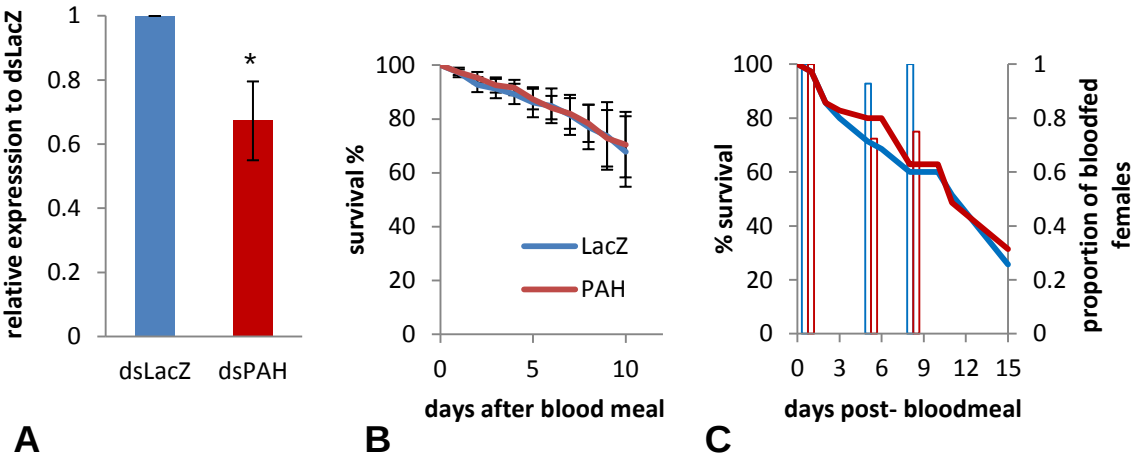


Fig. 3.6: Knockdown of PAH does not affect the survival of blood-fed *An. gambiae* mosquitoes. **A** Q-PCR showing the effect of dsPAH injection on the mean PAH mRNA abundance in dsPAH injected 24h postblood-fed *An. gambiae* mosquitoes relative to the dsLacZ controls (delta- delta Ct method). The endogenous reference gene was RPL19. The error bars represent the standard error of the mean (4 independent experiments). Paired t-test: * $p<0.05$ **B** Mean survival of dsLacZ and dsPAH injected mosquitoes after blood-meal of 4 independent experiments. The error bars represent the standard error of the mean (log-rank test for each independent experiment $p>0.05$). **C** The effect of 3 consecutive blood meals on the survival of 30 dsPAH or dsLacZ injected females (log-rank test $p>0.05$).

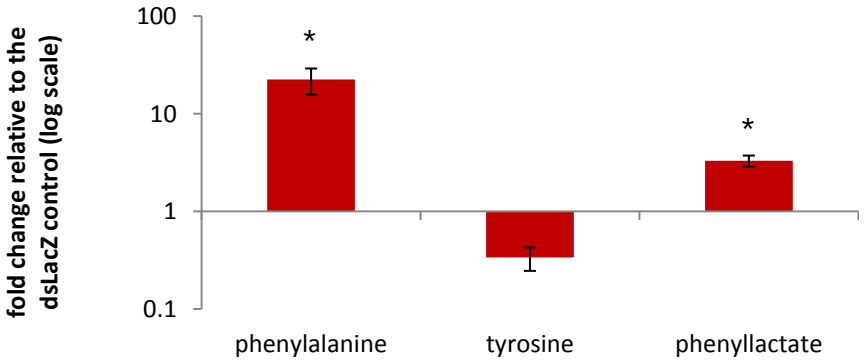


Fig. 3.7: GC-MS analysis of key amino acids in the phenylalanine metabolism in response to silenced PAH. Fold change in the mean phenylalanine, phenyllactate and tyrosine concentration from dsPAH injected mosquitoes compared to the dsLacZ injected controls 24h post-blood meal. The vertical lines represent the standard error of the mean of 3 different experiments (paired t- test on log- transformed data: * $p<0.05$).

3.5. The knockdown of PAH reduces the melanisation response in *P. berghei* infected *An. gambiae* mosquitoes

A PAH knockdown was shown to lead to a reduced tyrosine formation in the mosquito body in response to a blood meal. The lack of tyrosine could have affected further the melanin synthesis involved in the mosquitos' innate immunity against parasites. Since any change to parasite transmission caused by interventions is of interest, this possibility was investigated by infecting dsLacZ or dsPAH injected mosquitoes with the rodent malaria *Plasmodium berghei*. The survival was recorded from the time of infected blood meal until mosquito dissection 8 days post blood meal. No difference in survival rate was found between the different groups in three different experiments (log-rank test, $p>0.05$) (**Figure 3.8A**).

8 days after the blood meal 88-90% of the surviving 103 dsPAH and 139 dsLacZ injected mosquitoes harboured at least one *P. berghei* oocyst (χ^2 –test, $p= 0.78$) (**Figure 3.8B**). There was no difference in the average total number of oocysts between dsPAH (55) and dsLacZ (58) infected mosquitoes in all three experiments (Mann- Whitney test, $p>0.05$) (**Figure 3.8C**).

Of the 92 dsPAH infected mosquitoes 89 carried GFP oocysts (97%) and 39 showed melanised oocysts (42%). In contrast to this, of the 131 dsLacZ infected survivors 126 harbored GFP oocysts (89%) and 88 carried melanised oocysts (59%).

The mean number of GFP oocysts per individual did not differ between the control (55) and dsPAH (49) injected group (Mann-Whitney, $p=0.67$) and large variations of the number of GFP oocysts (0-420 GFP oocysts) were found between individuals within a group. However, combining data from all three experiments, the degree to which oocysts were melanised by a mosquito, varied significantly between dsPAH (15%) and dsLacZ (28%) injected mosquitoes (t-test of the arcsine transformed proportion of melanised oocysts, $p=0.004$) (**Figure 3.8D**). This means that the knockdown of PAH has an effect on the ability of mosquitoes to melanise *P. berghei* parasites.

This result is in concordance with a study in *Aedes aegypti* which showed a reduced melanisation reaction in response to PAH knockdown (Infanger et al., 2004). In *Anopheles gambiae* no effect on the melanisation of injected abiotic sephadex beads upon knockdown of phenylalanine hydroxylase had been observed so far (Paskewitz and Andreev, 2008). This

difference can be possibly explained by the fact that melanisation of different targets are often controlled by different pathways even within species (Paskewitz and Andreev, 2008, Zheng et al., 2003, Tang et al., 2006). Further, a tissue-specific knockdown of PAH could possibly affect parasites located in the midgut, but leave for example beads that widely distributed in the haemocoel unaffected.

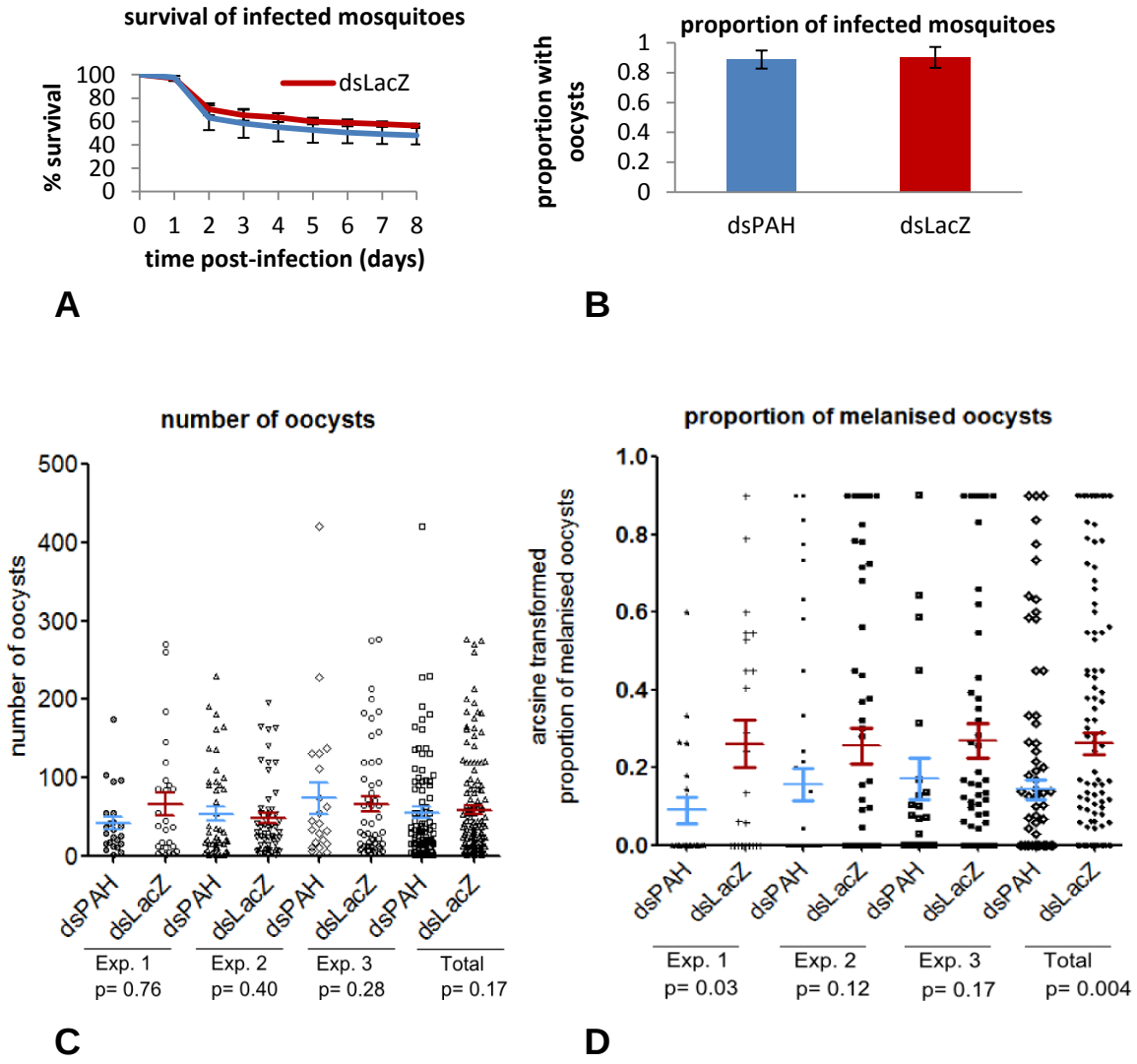


Fig. 3.8: PAH silenced females show a reduced melanisation response upon *P. berghei* infection. **A** Displayed is the mean and standard error of the mean of the survival of dsLacZ and dsPAH injected female adult mosquitoes after a *P. berghei* infected blood meal (Log-rank test 1. repeat p=0.03, 2. repeat p=0.31, 3. repeat p=0.57, overall p >0.05). **B** success of the infection by *P. berghei* in dsLacZ and dsPAH injected mosquitoes. The proportion of mosquitoes with a least one oocyst 8 days after blood feeding (χ^2 –test, p= 0.78) **C** Number of oocysts (melanised and non-melanised) in a single mosquito in 3 independent experiments. The horizontal lines show the mean and standard error of the mean (Mann-Whitney test, p<0.05 significant). **D** Mean proportion of oocysts melanised by a mosquito. The horizontal lines show the mean and standard error of the mean (t-test of the arcsine transformed proportion of melanised oocysts, p<0.05 is significant).

3.6. Effect of PAH knockdown on the reproductive potential and oviposition behaviour

Although, the reduced ability to melanise *Plasmodium* parasites had no effect on the mosquitos' survival, the reduced melanin synthesis could have further pleiotropic physiological effects such as reduced melanisation of the chorion which is essential to the egg development leading to the reduction in the mosquito reproductive potential.

In the following 3 independent experiments mated dsLacZ or dsPAH injected *An. gambiae* females were blood-fed and the number of eggs laid and the hatching rate was recorded. Interestingly, in all experiments fewer dsPAH injected blood-fed females were able to lay eggs. The remaining dsPAH injected ovipositing females, showed no difference in their fecundity (number of eggs per female) but compared to the dsLacZ injected control their fertility rate (hatching rate per ovipositing female) was significantly reduced (**Table 3.1).**

Because a knockdown of phenylalanine hydroxylase led to a reduced tyrosine concentration which is the precursor of dopa and the neurotransmitter dopamine it was assumed that possibly the mosquito behaviour could have been changed in response to this knockdown.

DsLacZ or dsPAH injected *An. gambiae* females were blood-fed and allowed to choose between 4 different coloured bowls (black, red, yellow, white) for oviposition (**Figure 3.9**). In three consecutive blood-meals no significant difference between their oviposition behaviour was found.

Therefore, a knockdown of PAH expression might have an effect on the ability of females to lay eggs and fertility rate, but not on the ability to distinguish different coloured oviposition surfaces.

Table 3.1.: Fecundity and fertility rates of mated dsPAH vs. mated dsLacZ injected female mosquitoes. Displayed is the number of dsRNA injected ovipositing females, median number of eggs and the hatching rate per ovipositing females from 3 independent experiments.

injection	no. of ovipositing females (arcsine transformed %)	p-value (t-test of arcsine transformed percentage of ovipositing females)	Median no. of eggs per female ±SEM	p-value (Mann-Whitney-test comparing the median number of eggs)	average hatching rate in % ±SEM	p-value (t-test of arcsine transformed hatching rate)
dsLacZ	29 (64% ±4*)	0.03	56±18	>0.05	67 ± 1*	0.02
dsPAH	21 (45% ±4*)		50±16		57 ± 2*	

*arcsine transformed data

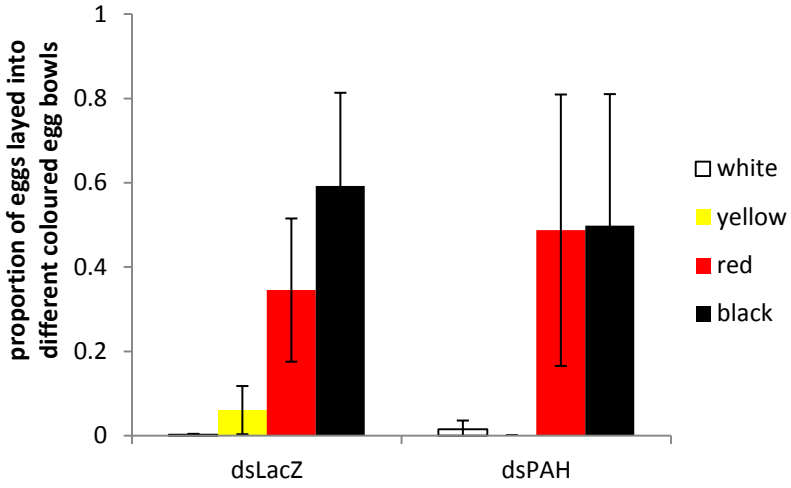


Fig. 3.9: DsPAH vs. dsLacZ injected mosquitoes do not differ in their chromatic oviposition behaviour. Proportion of eggs laid into different coloured egg bowls per 10 blood-fed females in 3 consecutive gonotrophic cycles (χ^2- test, p=0.99).

3.7. The immune response related gene Pro-phenoloxidase 9 (PPO9) is highly upregulated in response to blood meal

Initially phenylalanine is hydroxylated to tyrosine by PAH, which is the rate limiting substrate for melanin synthesis (Johnson et al., 2003). A knockdown of PAH was shown to reduce the amount of tyrosine found in mosquitoes and melanin synthesis. However, this reduction was relatively low (3-fold) compared with the increase in phenylalanine (22-fold). One reason for this could be insufficient knockdown of PAH by dsPAH RNA; another one is that tyrosine can be also ingested with the blood meal. This ingested tyrosine can thus provide melanin. It was therefore concluded that knocking down enzymes further downstream in that pathway needed for innate immunity could be more effective in completely inhibiting melanin synthesis.

Tyrosine can be converted to dopa and dopaquinone by phenoloxidase (PO). The term PO is often used to describe the tyrosinase-like reactions of enzymes that mediate the hydroxylation of monophenols and oxidation of derived *o*-diphenols (Christensen et al., 2005a). These POs are synthesised as inactive prophenoloxidases that are activated by a serine protease-activating enzyme in response to injury or infection. The *An. gambiae* genome contains nine putative PPOs. In comparison with this, *D. melanogaster* has only three PPOs (Asada et al., 1999). The importance of PPO polymorphisms in mosquitoes is unknown (Christensen et al., 2005b). In *An. gambiae* blood meals have shown to induce the expression of PPO1, PPO4 and PPO9 genes and downregulation of PPO5 (Mueller et al., 1999; Christophides et al., 2004).

In this project, the role of PPO9 was investigated because it was previously shown to have the highest transcript level in response to blood meal suggesting an important function for immunity, reproduction or amino acid metabolism (Christophides et al., 2004).

In the following single experiment it was confirmed that the expression of the single PPO9 transcript was highly upregulated in all tissues and peaked in the carcass and head 24h post-blood meal (PB), in the midgut and ovaries 48h PB (**Figure 3.10**). Overall PPO9 was stronger upregulated than PAH in response to a blood meal, indicating that a knockdown in PPO9 could have potentially a stronger physiological effects than PAH.

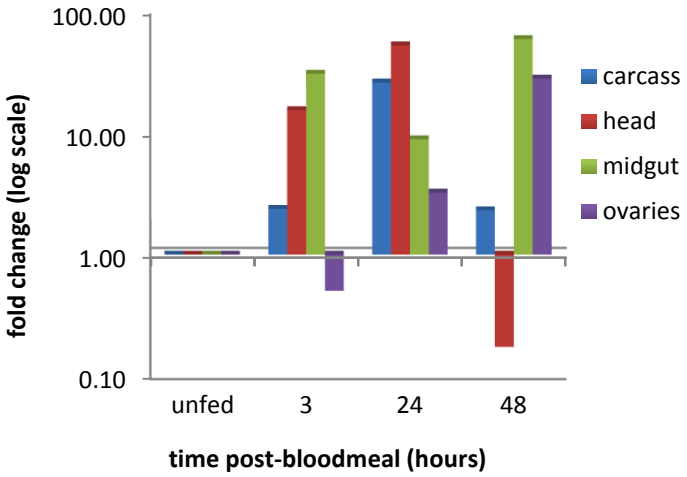


Fig. 3.10: Prophenoloxidase 9 (AgPPO9) expression in different female *An. gambiae* mosquito tissues in response to blood meal. The tissue-specific PPO9 expression at time 0 was used as reference. The endogeneous control was the ribosomal gene *RPL19*.

3.8. **A knockdown of PPO9 reduces mosquito survival**

In insects phenoloxidases have been shown to play an important role in the conversion of tyrosine to dopa and dopaquinone, the dopamine and melanin precursor. In *Drosophila melanogaster* two of three prophenoloxidases have to be shown to be essential for its survival (Asada et al., 1999). In *Anopheles gambiae* prophenoloxidase 9 (PPO9) showed a high upregulation after a blood meal which contains the sole source of phenylanine and tyrosine in adult *An. gambiae* mosquitoes. When PPO9 was targeted by dsRNA, compared to the knockdown of PAH (33%), the PPO9 transcript level was reduced on average by 77% (**Figure 3.11A**). The survival analysis of dsPPO9 or dsLacz injected mosquitoes showed a significant increase in the mosquito mortality mainly 24h post- blood meal (log-rank test, $p<0.05$) (**Figure 3.11B**). This time coincided with the digestion of blood-meal proteins and blood-meal dependent expression pattern of PPO9 (Jahan et al., 1999, Marinotti et al., 2005). The following days mosquitoes returned to their sugar based diet, which contained no protein and thus required no increased PPO 9 activity for mosquito survival.

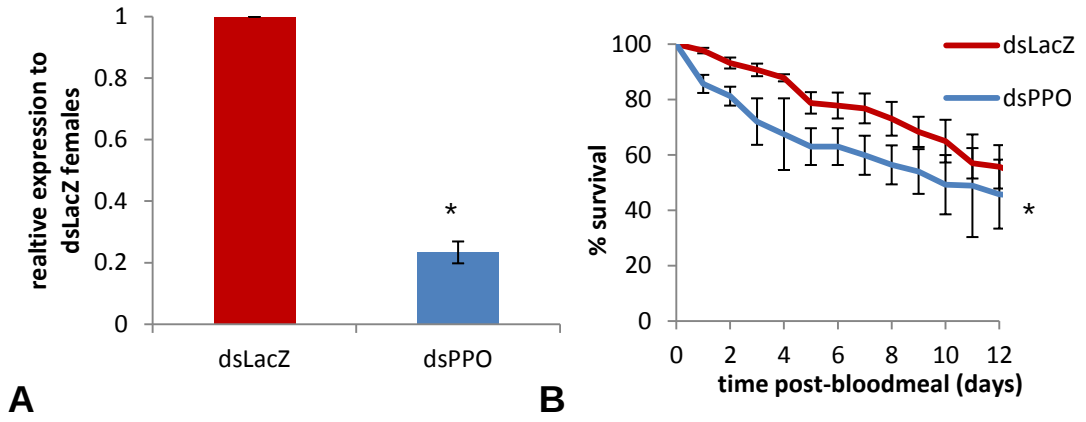


Fig. 3.11: Knockdown of PPO9 reduces the survival of blood-fed *An. gambiae* mosquitoes. A: Q-PCR showing the mean PPO9 mRNA abundance in dsPPO injected blood-fed *An. gambiae* mosquitoes relative to the dsLacZ controls (delta- delta Ct method). The endogenous reference gene was RPL19. The vertical lines represent the standard error of the mean. Paired t-test: * $p<0.05$ **B**: Displayed is the mean and standard error of the mean of the survival of dsLacZ and dsPPO injected mosquitoes after blood-meal from 3 different experiments. Log-rank test: 1. repeat p= 0.39, 2. repeat p= 0.005, 3. repeat p=0.38, overall * p =0.007.

3.9. PPO9 knockdown reduces the number of oocysts independent of the melanisation response in *P. berghei* infected mosquitoes

In three experiments a total of ~ 300 mosquitoes were injected either with dsLacZRNA or dsPPO RNA and offered a blood meal 4 days post injection. 8 days after blood meal 69 dsLacz and 78 dsPPO9 injected mosquitoes were dissected and the number of occysts counted for each mosquito. In both groups over 91% of the mosquitoes were successfully infected (**Figure 3.12 A**). The survival of dsPPO9 injected mosquitoes was not significantly reduced after an infected blood meal (**Figure 3.12 B**). There was no difference in the number of occysts between dsLacZ and dsPPO9 injected mosquitoes (Mann Whitney test, $p>0.05$) (**Figure 3.12 C**). Interestingly, in contrast to the previous PAH knockdown and infection experiment, the proportion of melanised oocysts to the number of total oocysts did not differ (**Figure 3.12 D**). Therefore, PPO9 did not affect as predicted melanin formation.

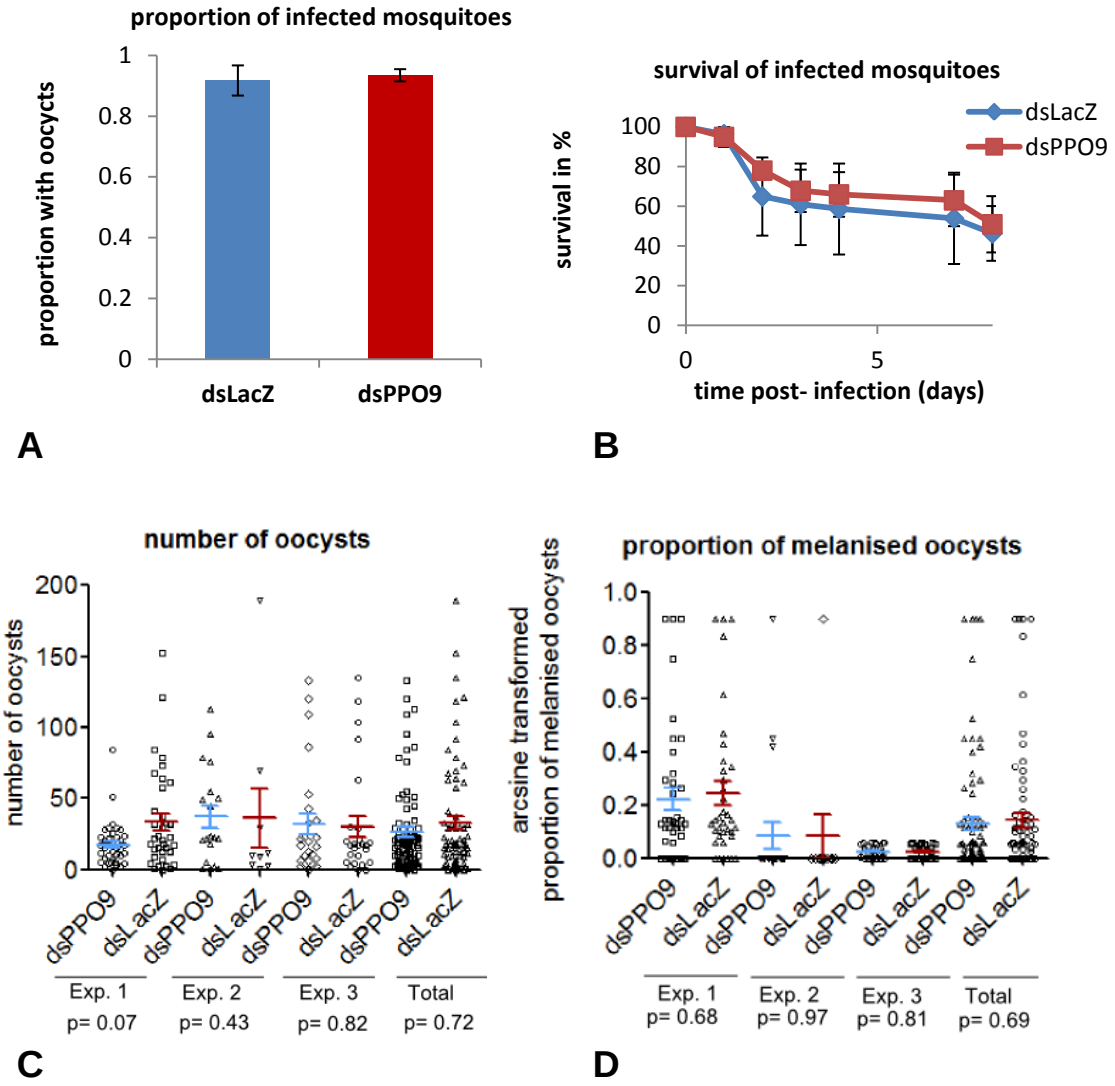


Fig. 3.12: PPO9 silenced females show a reduced oocyst formation upon *P. berghei* infection. A success of the infection by *P. berghei* in dsLacZ and dsPPO injected mosquitoes. The proportion of mosquitoes with a least one oocyst 8 days after blood feeding (average and SEM of 3 independent experiments) **B** Displayed is the survival of dsLacZ and dsPPO injected female adult mosquitoes after a *P. berghei* infected blood meal. Log-rank test: 1. repeat p=0.08, 2. repeat p=0.04, 3. repeat p=0.8, overall p=0.64). **C** Mean number number of oocysts in a single mosquito (Mann Whitney test, p=0.72) **D** Mean proportion of oocysts melanised by a mosquito (t-test of arcsine transformed proportion of melanised oocysts, p<0.05 significant). The horizontal lines represent the standard errors of the means.

3.10. **A knockdown of PPO9 does not affect the reproduction potential or oviposition behaviour.**

Although PPO9 might not be needed for the melanotic encapsulation response against *P. berghei*, its upregulation in response to blood meal suggests that it might play a role for oviposition like the melanisation of the egg chorion which has been observed in *Aedes aegypti* (Kim et al., 2005). 20 single females were placed each into oviposition cups and their number of eggs and larval hatching rate was recorded. Out of the 7-8 females which laid eggs no difference in the average number of eggs or larval hatching rate was found (**Table 3.2**). Both, the oviposition assay and the previous infection assay suggest that PPO9 is not involved in these major insect melanisation processes.

In order to elucidate its potential function in regulating dopamine or other neurotransmitters, the chromatic visional ability was investigated. Although more dsPPO9 injected mosquitoes were found to prefer red oviposition bowls (χ^2 –test, $p<0.05$) in general these mosquitoes were able to distinguish between different coloured egg bowls implicating no change in their visual ability due to lack of dopamine (**Figure 3.13**).

Table 3.2: Reproduction potential of dsPPO9 vs. dsLacZ injected female mosquitoes.

injection	no. of females laying embryonated eggs (%)	average no. of eggs ±SEM	p-value (Mann Whitney test comparing number of eggs of dsLacZ vs. PPO9)	average hatching rate in % ±SEM	p-value (t-test of arcsine transformed hatching rate of dsLacZ vs. dsPPO9)
dsLacZ	8 (40%)*	95.75±11.99	0.22	52.97±0.10	0.94
dsPPO9	7 (35%)*	76.29±12.30		52.80±0.05	

* out of total of 20 females

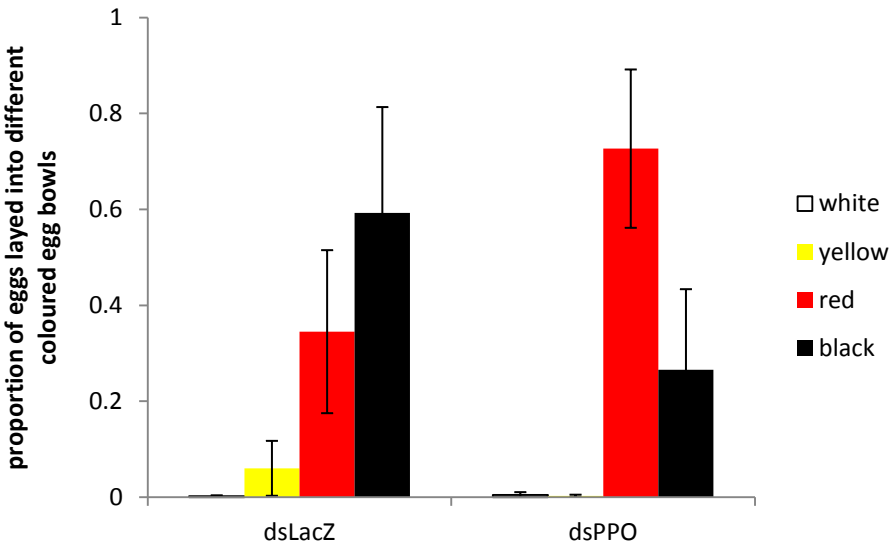


Fig. 3.13: DsPPO9 silenced mosquitoes can distinguish between differed coloured egg bowls. Proportion of eggs laid into different coloured egg bowls by 10 blood-fed females in 3 consecutive gonotrophic cycles. χ^2 –test comparing the proportion of eggs laid into red oviposition bowls between dsPPO9 and dsLacZ (control) showed a significant difference ($p<0.05$).

3.11. **Investigation of dopa decarboxylase or tyrosine hydroxylase activity in *An. gambiae***

Two other enzymes believed to be involved in the phenylalanine/tyrosine metabolism in *An. gambiae* are tyrosine hydroxylase (TH) and dopa decarboxylase (DDC). Like phenoloxidase, tyrosine hydroxylase (AGAP006023) is predicted to convert tyrosine to dopa in mosquitoes. However, little is known about tyrosine hydroxylase and its role in melanisation. Tyrosine hydroxylase- dependent dopamine formation has been reported in *D. melanogaster*, leading to reduction in locomotory behaviour but to date no studies have addressed the role of this enzyme in mosquitoes (Riemensperger et al., 2011). In contrast to this, dopa decarboxylase (AGAP009091), which is responsible for the conversion of dopa to dopamine has been characterised in mosquitoes. In an example a double subgenomic *Sindbis* virus was used to silence DDC and assess its role in melanisation of microfilariae in the mosquito species *Armigeres subalbatus*. DDC-silenced mosquitoes exhibited a decreased melanisation ability and high mortality, over-feeding and abnormal movement, consistent with an involvement of DDC in neurotransmission (Huang et al., 2005). In another study, Paskewitz and Andreev targeted DDC in *Anopheles gambiae* and found an effect in the melanisation response (Paskewitz and Andreev, 2008). However, a blast analysis of the primers used for the dsRNA synthesis revealed that the sequences belonged to the PO activating Clip domain serine protease subfamily B (ClipB9) (AGAP003058) instead of DDC (AGAP009091). It is therefore unclear whether the ClipB9 or DDC was silenced.

In the following experiment, the expression of TH and DDC was found to be upregulated in response to blood meal. In fact, TH and DDC enzymes were expressed in markedly similar fashion, showing for example a single high peak of expression in the head and carcass 3h post-blood meal and upregulation in the midgut and ovaries consistent with a role for these two enzymes in the same metabolic pathway (**Figure 3.14A, B**). Unfortunately, an RNAi mediated knockdown of these two enzymes was not detected (**Figure 3.14C, D**). In order to investigate whether dsRNA injection has a phenotypic effect on *An. gambaie* mosquitoes their life span was recorded. However, neither dsTH nor dsDDC injection reduced the mosquito survival (**Figure 3.14E, F**). This suggested that possibly no knockdown of either of the two enzymes had been achieved. In line with this, in general tissues such as the ovaries can be less accessible for dsRNA compared to the rest of the carcass which might prevent a successful knockdown of transcripts in these tissues (Ciudad et al., 2007).

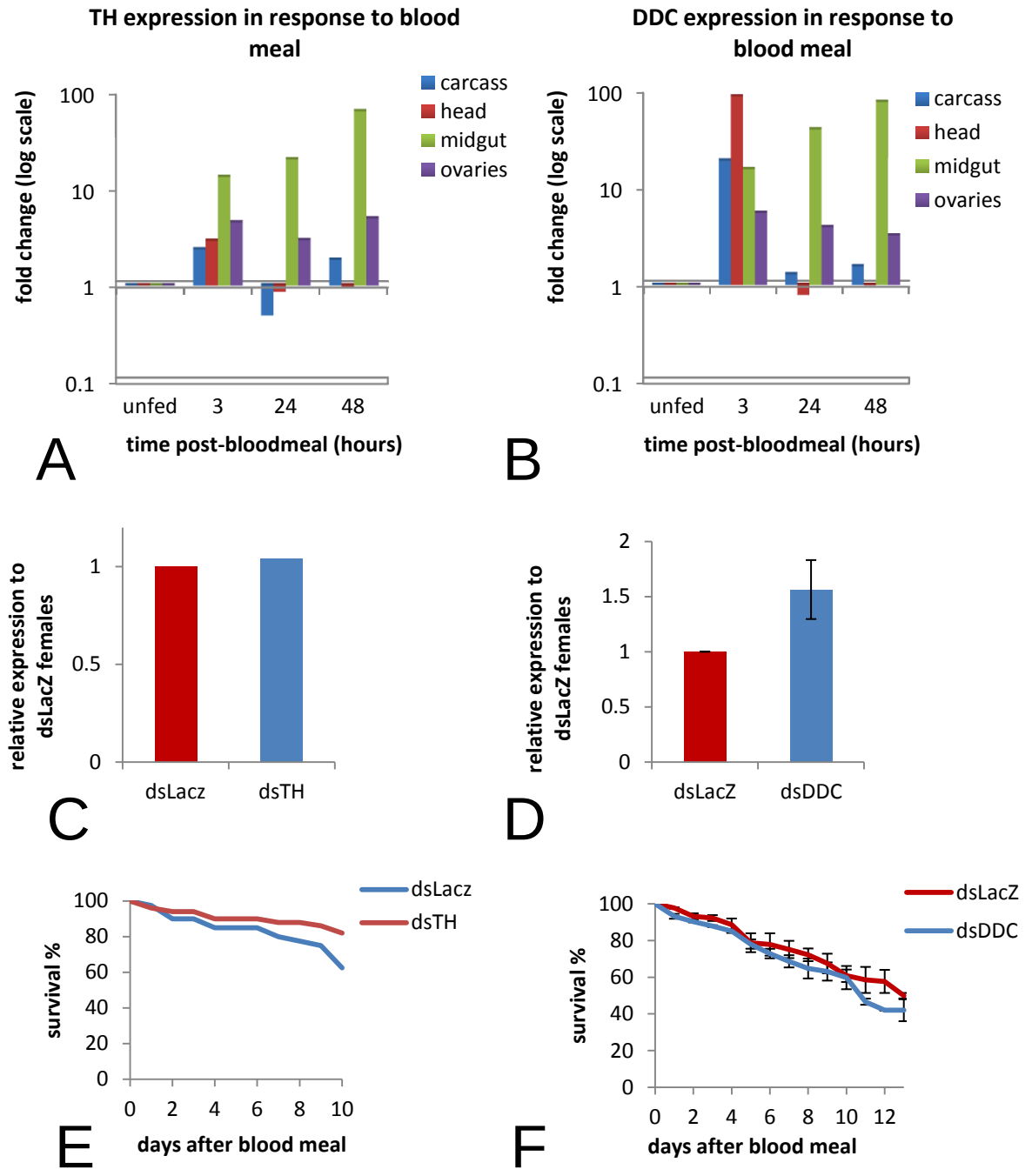


Fig. 3.14: TH and DDC expression analysis in adult blood-fed female *An. gambiae* mosquitoes. A Tissue specific expression of tyrosine hydroxylase (TH) in response to blood meal **B** Tissue specific expression of tyrosine hydroxylase (TH) in response to blood meal **C** Q-PCR showing the TH mRNA abundance in dsTH injected blood-fed *An. gambiae* mosquitoes relative to the dsLacZ controls (delta-delta Ct method). The endogenous reference gene was RPL19. **D** Q-PCR showing the mean DDC mRNA abundance in dsDDC injected blood-fed *An. gambiae* mosquitoes relative to the dsLacZ controls (delta- delta Ct method). The endogenous reference gene was RPL19. The vertical lines represent the standard error of the mean (5 repeats). **E**: Survival curve of dsLacZ and dsTH injected mosquitoes after blood meal. Log-rank test: $p>0.05$ **F** Displayed is the mean and standard error of the mean of the survival of dsLacZ and dsDDC injected mosquitoes after blood-meal from 3 independent experiments (log-rank test: 1. repeat $p=0.21$, 2. repeat $p=0.29$, 3. repeat $p=0.55$, overall $p>0.05$).

4. Chapter: Manipulation of the phenylalanine/tyrosine metabolism in *An. gambiae* – Discussion

Some pathways like the metabolism of phenylalanine/tyrosine are highly conserved between human and *An. gambiae*. Because in humans disruptions in this pathway often cause severe brain damage one strategy employed in this project was to knockdown key enzymes in this pathway which could lead to death in response to an amino acid- rich blood meal.

Feeding mosquitoes with high concentrations of phenylpyruvate (PPA), the toxic accumulation product in PKU patients, led to a high increase in phenyllactate (PLA) and premature death. The toxic effect of PPA/PLA occurred faster when PPA was administred with blood than with glucose. One reason for this could be that the administered glucose potentially compensated the inhibiting effect of phenylpyruvate in glucose metabolism (Gazit et al., 2003b). Another reason is that the uptake of glucose and blood is different in mosquitoes, while glucose is taken up in the crop; blood is ingested in the midgut, exposing the mosquito more rapidly to PPA.

The dsRNA mediated knockdown of the *Anopheles gambiae* PAH did not lead to a large increase in phenyllactate and decrease in life span even after repeated blood meals. Reasons for this could be various. Firstly, no complete knockdown of PAH was achieved which allowed its metabolisation product tyrosine instead of phenyllactate. Even in humans only patients with a phenylalanine hydroxylase activity of 10% or less show classic phenylketonuria (Okano et al., 1991). Secondly, the amount of phenylalanine ingested with a blood meal was possibly too low to produce sufficient amount of phenyllactate to be toxic for the mosquito. While the latter point cannot be modified due to the nature of the mosquito, possibly the knockdown of PAH could be improved by injecting either a higher concentration of dsRNA, by heritable gene silencing from stably expressed transgenes or by using another system like the Sindbis virus that has been used to introduce dsRNA into *Anopheles* mosquitoes (Huang et al., 2005, Tamang et al., 2004, Brown et al., 2003). Possibly a knockdown of other enzymes involved in the degradation of other amino acids like alanine, glycine, valine and lysine which have a more than 3 fold higher concentration than phenylalanine in the blood might be more suitable to induce an aminoacid disease in

mosquitoes. Some human diseases which are characterised by severe mental defects caused by malfunctioning enzymes in these pathways have homolgous enzymes in *An. gambiae* which are upregulated in response to blood meal (**Table 4.1**).

Table 4.1: Human diseases caused by malfunctiong enzymes involved in valine, lysine and glycine metabolism

amino acid	Human disease	severe symptoms in humans	gene	homologous gene in *An. gambiae* mosquitoes (% identity using NCBI)	Fold change in gene expression post blood meal in An. gambiae mosquitoes (Marinotti et al., 2005) -2 0 +3		
					3h PBM	24h PBM	96h PBM
lysine	Glutaric academia	neuronal damage (Funk et al., 2005)	glutaryl-CoA dehydrogenase (GCDH)	AGAP000851 (59%)			
lysine	Saccharopinuria	spastic diplegia possible (Simell et al., 1972)	saccharopine dehydrogenase (SCCPDH)	AGAP002652 (44%)			
				AGAP003322 (41%)			
valine	Maple syrup urine diseas	mental retardation (Kalyanaraman et al., 1972)	branched-chain alpha-keto acid dehydrogenase (BCKDHA)	AGAP003136 (59%)			
glycine	Sarcosinemia	mild retardation (Sewell et al., 1986)	sarcosine dehydrogenase (SARDH)	AGAP007123 (51%)			
glycine	D- Glyceric acidemia	mental retardation, seizures (Duran et al., 1987)	aminomethyl-transferase (AMT)	AGAP001124 (54%)			
			glycine dehydrogenase (GLDC)	AGAP003321 (49%)			
glycine	Glutathione synthetase deficiency	mental retardation (Marstein et al., 1976)	glutathione synthetase (GSS)	AGAP000534 (44%)			

Even if the phenylalanine metabolism might not have been suitable for inducing an aminoacid disease by accumulation of toxic by-products, its proposed involvement in other life processes such as innate immunity and behaviour are important for malaria transmission and required further investigation.

Upon infection with *P. berghei*, a reduced ability to melanise parasites at the ookinete stage was observed for dsPAH- injected mosquitoes. These experiments showed for the first time that phenylalanine hydroxylase is required for the *An. gambiae* melanisation response after *P. berghei* infection. Previously, Paskewitz and colleagues showed that dsPAH injected *An. gambiae* mosquitoes did not have a reduced ability to melanise inoculated abiotic targets (Sephadex beads) (Paskewitz et al., 2008). In general it is believed that oocysts and beads melanise at the same proportion due to shared genetic mechanism of melanisation (Gorman et al., 1996). However, these two different results imply that the melanisation response might be controlled by PAH and PAH independent pathways depending on the infection agent. Further, in a field study it was found that 90% of field-captured mosquitoes melanised C-25 Sephadex beads, but only 2 out of 431 infected mosquitoes harboured melanised oocysts (Schwartz and Koella, 2002).

It is important to note that mosquitoes with an impaired melanisation activity could possibly give the parasite an advantage which could lead to an increase in its transmission potential. However, some researchers have argued that melanisation is unlikely a major factor in natural resistance to the major human parasite *P. falciparum* (Schwartz and Koella, 2002). Michels and colleagues strengthened this view by showing that by knocking down the serpin gene *SRPN2*, a proposed negative regulator of the melanin synthesis, the increasing melanisation response in *Anopheles gambiae* did not affect the *Plasmodium falciparum* development (Michel et al., 2006). The authors suggested that this might be caused by immune evasion strategies of the human parasite.

The knockdown of PAH in infected females showed further no effect on their life span. Possibly the knockdown was not sufficient to cause a decrease in survival or the melanisation of *P. berghei* was not essential for the adult mosquito survival. This would be in accordance with a study by Schnitger et al. which observed that mosquitoes did not require the melanisation response to survive a bacterial infection (Schnitger et al., 2007).

Because melanin is not only needed for the immune response and wound healing but also for the cuticular sclerotisation involved in hardening of the egg chorion, the effect of PAH

knockdown rates on oviposition and hatching rate were established (Sugumaran, 2002, Christensen et al., 2005b). In three independent experiments, only 39-52% of the females (total number of 40) laid eggs, compared to 59-72% (total number of 36) of the control injected with dsLacZ. Of the ovipositing females, the number of eggs and the larval hatching rate did not differ between both groups. One reason of why fewer females laid eggs could be that the phenylalanine/tyrosine availability also controls dopamine (DA) and octopamine synthesis, the latter has been shown to be a regulator of oviposition in *D. melanogaster* (Monastirioti et al., 1996). After phenylalanine is hydroxylated to tyrosine, the enzyme tyrosine hydroxylase or phenoloxidase catalyses the conversion of tyrosine to L-DOPA which is then either transformed to dopaquinone, a precursor of melanin, to tyramine the precursor of octopamine or decarboxylated by dopa decarboxylase to dopamine (DA) (Christensen et al., 2005). Monastirioti showed that a knockout of the tyramine beta hydroxylase causes a lack of octopamine which allowed females to mate and to develop eggs, but did not lead to a release of these eggs. Feeding of octopamine reversed this effect. In order to investigate this possibility in the future gravid dsPAH injected mosquitoes could be fed with octopamine. Further, a lack of dopamine can be associated with the oviposition arrest observed in these dsPAH injected females. Gruntenko and colleagues observed that DA decreased the juvenile hormone (by stimulating its degradation and inhibiting its synthesis) level in sexually mature flies (Gruntenko and Rauschenbach, 2004, Gruntenko et al., 2005). It was concluded that decreases in dopamine would be expected to increase the juvenile hormone titre which has been shown to cause oviposition arrest in flies and which was possibly observed in this experiment (Gruntenko and Rauschenbach, 2008).

Another pleiotropic effect that might be associated with reduced melanin or dopamine is a reduced visual and locomotory ability which has been observed in homozygous *ebony* fly mutants lacking the beta alanyl dopamine synthetase (Wittkopp et al., 2002, Borycz et al., 2002, Newby and Jackson, 1991). Further PAH defective *D. melanogaster henna* mutants show defects in their eye pigmentation.

In order to investigate the mosquito visual ability, gravid dsPAH and mock injected mosquitoes were allowed to choose to lay their eggs in low to high intensity coloured egg bowls. No reduced ability to distinguish the different colours was observed which showed that the PAH dependent melanin/dopamine synthesis is possibly not needed in fully developed adult eyes.

Although the PAH knockdown caused a reduced parasite melanisation, the melanin response was not completely inhibited. One reason for this could be the insufficient knockdown of PAH by dsPAH RNA. Another potential reason is that tyrosine, the PAH product can be also ingested with the blood meal and thus provide melanin precursors. It was therefore concluded that knocking down enzymes further downstream in that pathway needed for innate immunity could be more effective to inhibit the melanin/dopamine synthesis.

There are two enzymes which can convert tyrosine to dopa, one is a phenoloxidase (PO), and the other one is tyrosine hydroxylase. Attempts to knockdown the tyrosine hydroxylase AGAP006023 in blood-fed female *An. gambiae* mosquitoes were unsuccessful. One reason for this could be that 24h post blood meal tyrosine hydroxylase was mainly upregulated in the ovaries which has been observed to be difficult to penetrate by dsRNA (Ciudad et al., 2007, Belles, 2010).

The knockdown (KD) of PPO9, one of the nine *An. gambiae* prophenoloxidases was successful and had an effect on the mosquito life span. Apart from a reduction in melanin and dopamine synthesis, other causes could have led to this effect such as the production of melanotic precursors, reactive oxygen species and nitrogen which are generated during melanogensis and are highly cytotoxic for parasites and possibly for the mosquito itself (Christensen et al., 2005b, Kumar et al., 2003).

Because prophenoloxidases have been shown to play a major role for the melanisation immune response in many different insect species, it was assumed that a knockdown of PPO9 would lead to a reduced melanisation of *P. berghei* oocysts (Christensen et al., 2005b). However, no difference in the melanisation between the dsPPO9 and control injected mosquitoes was found. This result suggested that in *An. gambiae* prophenoloxidases might be involved in other melanin independent pathways. This is supported by another study which found that for example PPO6 is not involved in melanisation of Sephadex beads although it is highly upregulated in response to their inoculation (Warr et al., 2006).

Recently, Rund et al. discovered that the prophenoloxidase (PPO)-encoding genes PPO6 (AGAP004977), PPO5 (AGAP012616), and PPO9 (AGAP004978) are not only upregulated upon blood meal they are also expressed in a circadian pattern along with other genes that cover diverse biological processes such as transcription/translation, metabolism, detoxification, olfaction, vision, cuticle regulation, and immunity (Rund et al., 2011). It will

be most interesting to uncover the role of PPOs' in these processes, which might uncover new targets to manipulate the vectorial capacity.

Lastly, instead of using RNAi to knockdown important genes in the phenylalanine/tyrosine pathway another possibility to shorten the adult mosquito life span will be to overexpress genes in this pathway. Recently, serpin-2 (SRPN) was identified as a key negative regulator of melanisation by inhibiting a clip-serine proteinase CLIPB9 that leads to activation of PPOs (Michel et al., 2006). Interestingly a knockdown of SRPN did not only lead to an increase in melanisation but also to a reduced adult life span. A double knockdown of SRPN2 and CLIPB9 reversed this pleiotropic phenotype induced by SRPN2 silencing and no decrease in survival was observed (An et al., 2011). This interaction implies that in a transgenic model overexpression of CLIPB9 under a blood-meal inducible promoter could possibly recapitulate the effects seen with a SRPN-2 knockdown and reduce the adult female life span as well as increasing the melanisation response. The success of this system would depend on the degree of the life span reduction, melanisation upon parasite infection and effect on other fitness parameters such as competitiveness and reproduction.

5. Chapter: Inducing a polyglutamine disease in *Anopheles gambiae* mosquitoes- Results

The second approach used in this project was to increase adult mortality late in life by introducing a model of Huntington's disease (HD) in *An. gambiae*. This disease is caused by expanded polyQ stretches (>35 repeats) in the N-terminal fragment of huntingtin (htt) which cause protein aggregation and lead to selective neurodegeneration. The length of the polyglutamine repeats is positively correlated with the onset and severity of the disease. This is suitable for the project because the length of polyglutamine stretches can be potentially modulated to that extent that adult mosquitoes die after they have reproduced but before they are able to transmit the parasite. The toxic mechanisms of this disease have yet to be fully elucidated, but some HD characteristics such as accumulation of htt peptides and neurodegeneration have been been recapitulated in animal model systems like *Drosophila* by expression of human expanded huntingtin exon 1 fragments under regulation of neuron and photoreceptor-specific promoters (Marsh et al., 2000, Jackson et al., 1998, Steffan et al., 2001). Because the disease was induced in flies and other model organisms, it was assumed that the expression of expanded N-terminal huntingtin (exon1) would have potentially similar effects in a transgenic HD mosquito model.

5.1. Generation of polyglutamine containing httex1pQ transformation vectors

During the DNA replication of the httex1pQ transformation plasmids in *Escherichia coli*, the original $pCAG_{51}$ tract underwent expansion to 52 CAG repeats, while the tract containing $pCAG_{93}$ lost from 2 to 24 CAG repeats. Therefore the final plasmids were called httex1p-20Q, httex1p-52Q, httex1p-69Q and httex1p-91Q **(Figure 5.1A,B).** This instability might explain the polymorphic polyglutamine (polyQ) tract in many proteins of eukaryotes. Importantly, these mutations did not change the reading frame. The sequence of the whole *Asc*I-*Asc*I cloned region did not reveal any further mutations in any of the plasmids.

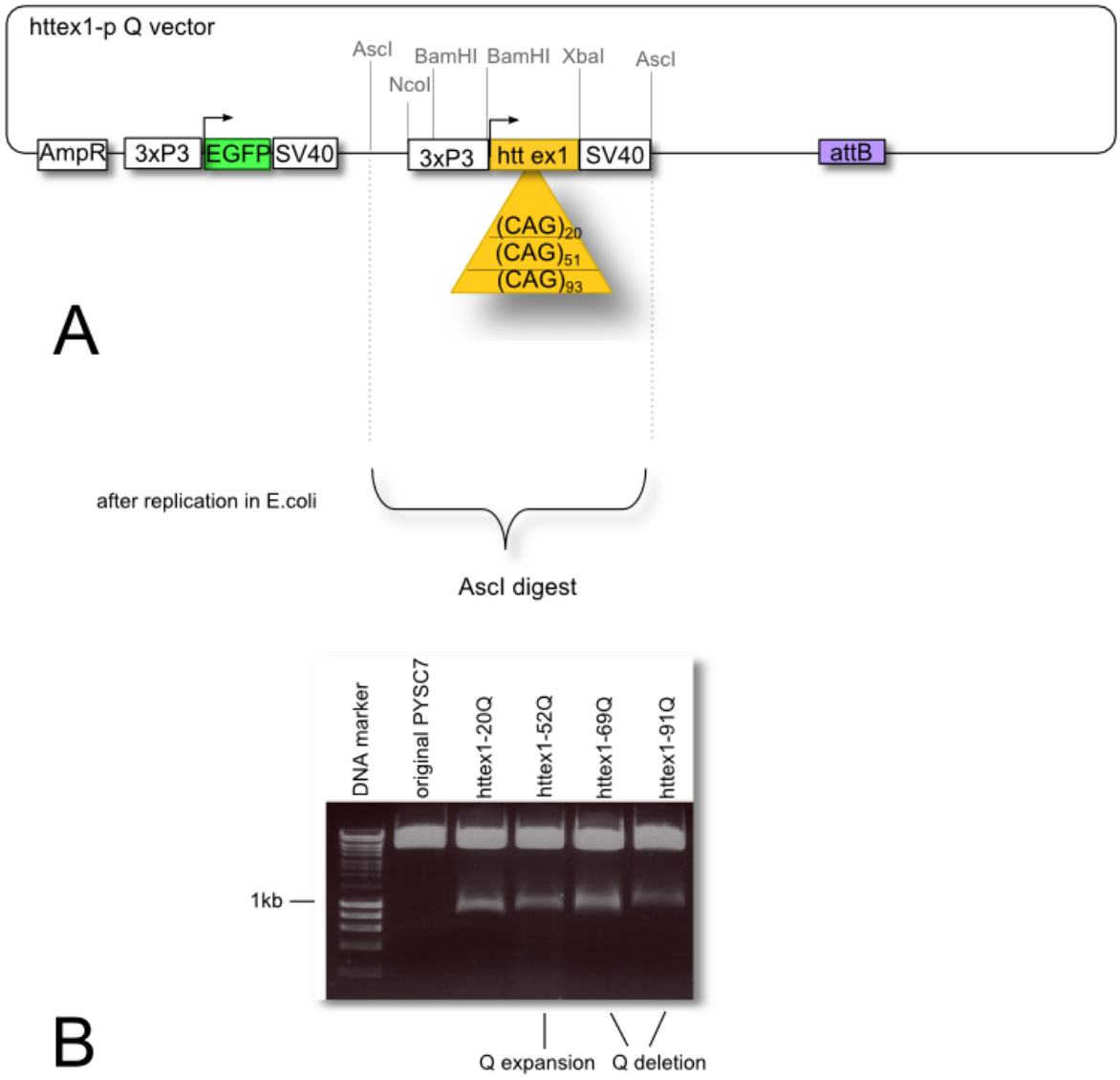


Fig. 5.1: Httex1pQ transformation vectors A Structure of the httex1p-*Q* vectors which included: *3x3P*- promoter, *SV40*- terminator, EGFP- marker gene, htt ex1 -*huntingtin* exon 1 containing various lengths of CAG (Q) repeats (20, 51,93), *attB*- insertion site of the *attP-attB* integration system, *AmpR-ampicillin resistance* gene, *AscI-XbaI* restriction sites used for cloning (shown not to scale) **B** Digest of the *AscI-AscI* fragment of the different httex1p-Q plasmids. The size of the *AscI-AscI* fragment differs in the number of polyQ repeats. During the DNA replication in *E. coli*, the original $polyQ_{51}$ tract underwent expansion to 52 polyQ repeats, while the tract containing $polyQ_{93}$ lost from two to 24 polyQ repeats.

5.2. **Establishment of the transgenic httex1pQ lines**

A site-specific *attB/attP* integration system was chosen for the insertion of the httex1pQ transformation vectors into *Anopheles gambiae attP* line E mosquitoes (**Figure 5.2A-C**) (Thorpe et al., 2000, Meredith et al., 2011). This non-random unidirectional integration requires an *attP* site in the genome (in this case *attP* line E) and a plasmid containing the *attB* site (the httex1pQ-transformation vector). The injected ΦC31 integrase mediates then the sequence-specific recombination between these two largely different attachment sites, *attB* and *attP*, which share a 3 bp central region, where the crossover occurs (Thorpe et al., 2000). The resulting 2 different *attL* and *attR* sites no longer serve as substrate for the Integrase and are therefore stable. This system also has the advantage over transposon mediated transformation systems that by integration into the same *attP* locus, it allows the direct comparison of different httex1pQ lines which differ only in their polyglutamine repeat length.

An overview of the injection results is shown in **Table 5.1** 6-18% of the injected *attP* line E embryos survived whereby 0.9-24% of the hatched larvae showed transient EGFP expression mainly in their abdomen. The surviving transient adults were outcrossed to wild type *An. gambiae* mosquitoes to check for germline integration and to follow the segregation of the marker. The progeny of the G_0 survivors contained 1.3-14% fluorescent individuals among the up to 2338 G_1 larvae analysed per injected plasmid. All of the EGFP expressing larvae were derived from only 1-2 transient male or female G_0 founders per experiment (**Table 5.1**). After the outcross of EGFP-expressing G_1 with the *attP* line E, all lines were intercrossed to make homozygotes. Throughout these the CFP gene (marking the *attP* genomic site) and the EGFP always co-segregated suggesting that the integration of the polyQ occurred at the *attP*-site (**Figure 5.3**).

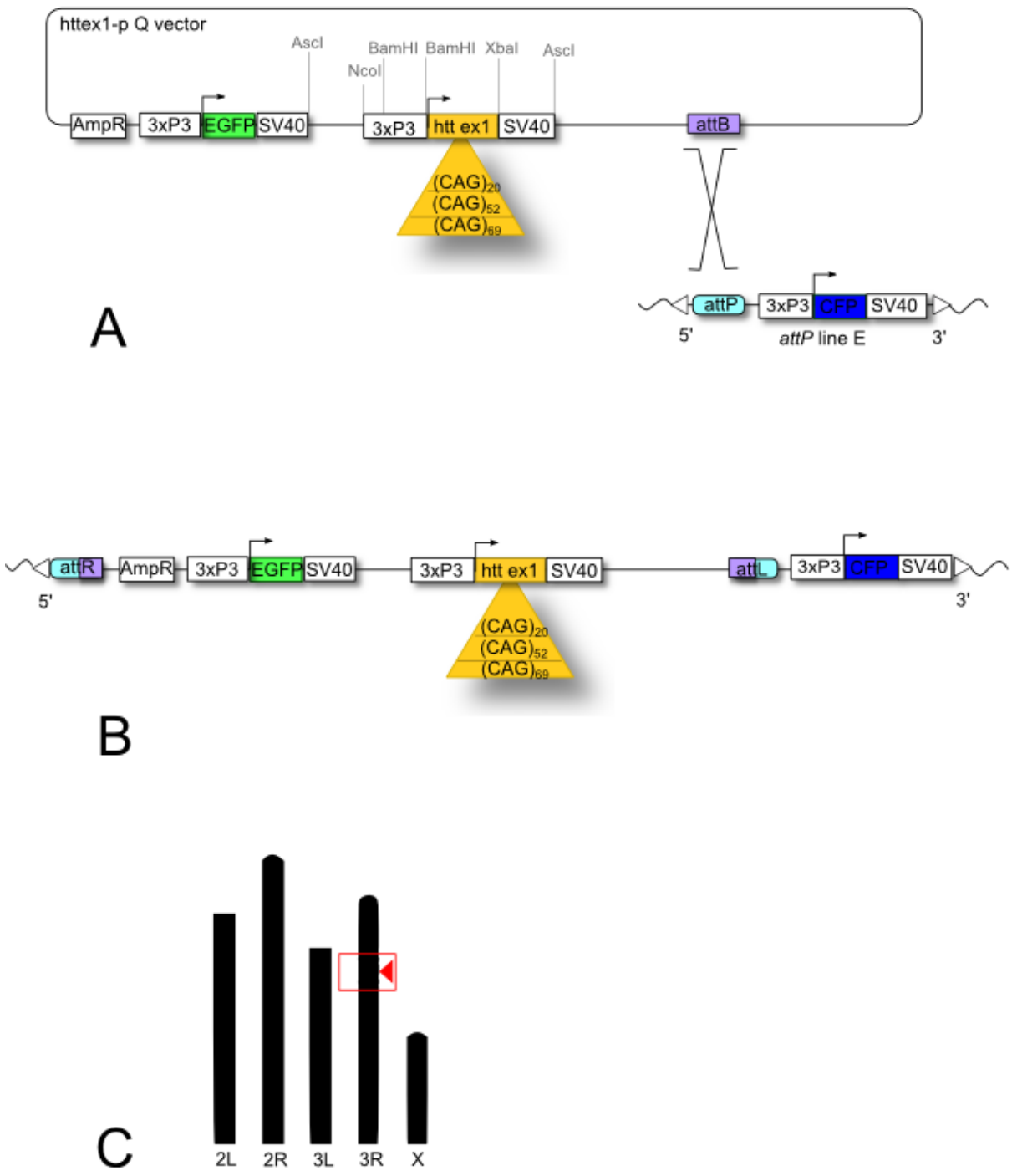


Fig. 5.2: The proposed mechanism of the attB/attP mediated integration of the httex1p vector into *attP* line E A Structure of the httex1p Q vector containing an *attB* site and the attP line E. *3x3P*-promoter, *SV40*- terminator, EGFP and CFP- marker gene, htt ex1 -*huntingtin* exon 1 containing various lengths of CAG (Q) repeats (20, 52, 69), *attB, attP*- insertion site of the *attP-attB* integration system, AmpR- *ampicillin resistance* gene **B** The crossover between the attB and attP sites forms *attL* and *attR* sequences flanking the integrated httex1pQ construct **C** Localisation of the *attP* site (site of integration) in the genome of the transgenic *An. gambiae attP* line E.

Table 5.1: Overview of the injections of different httex1p-Q plasmids into *An. gambiae attP* line E embryos

injected plasmid	injected embryos	hatched larvae	transient adults	crossing scheme	fluorescent/ total G1	transient G0 founder
httex1p52Q	1272	76 (6%)	18 (1.4%)	Wt outcross	48/345 (14%)	1 male
httex1p20Q	1571	255 (16%)	19 (1.2%)	Wt outcross	30/2338 (1.3%)	1male
httex1p69Q	1285	233 (18%)	2 (0.2%)	Wt outcross	33/280 (12%)	1 or 2 females

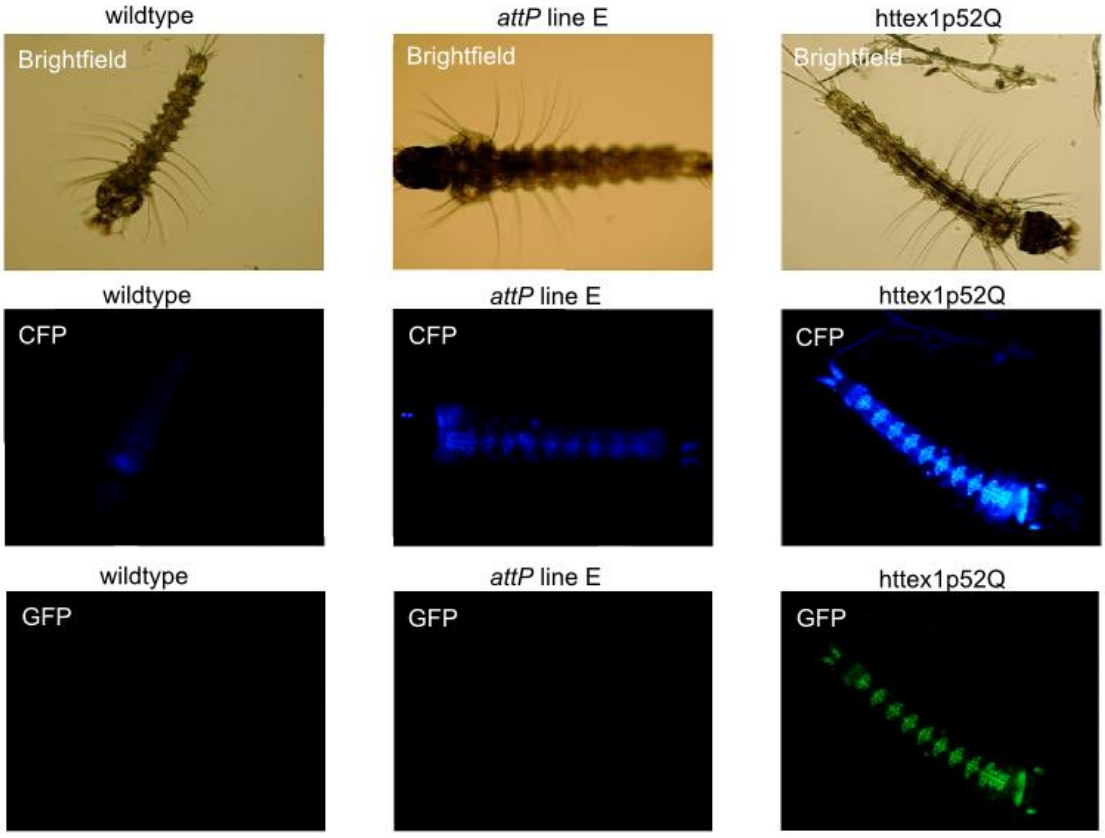


Fig.5.3: Identification of httextpQ transformed mosquitoes. Transgenic individuals of the httex1pQlines could be easily distinguished from wild type and *attP* line because of their characteristic strong expression pattern of EGFP in the brain, eye, ventral chord and anal papillae of larvae that is typical for the *3xP3* promoter.

5.3. Confirmation of the site-specific unidirectional httex1pQ integration

In all polyQ- transformed mosquitoes a close linkage of the EGFP (httex1pQ-specific) and CFP (*attP* line E-specific) expression was observed suggesting that a site-specific integration had occurred at the CFP marked *attP* locus. Molecularly, this was confirmed by genomic PCR using primers specific for the inserted httex1pQ construct and the specific integration site **(Figure 5.4 A-C)**.

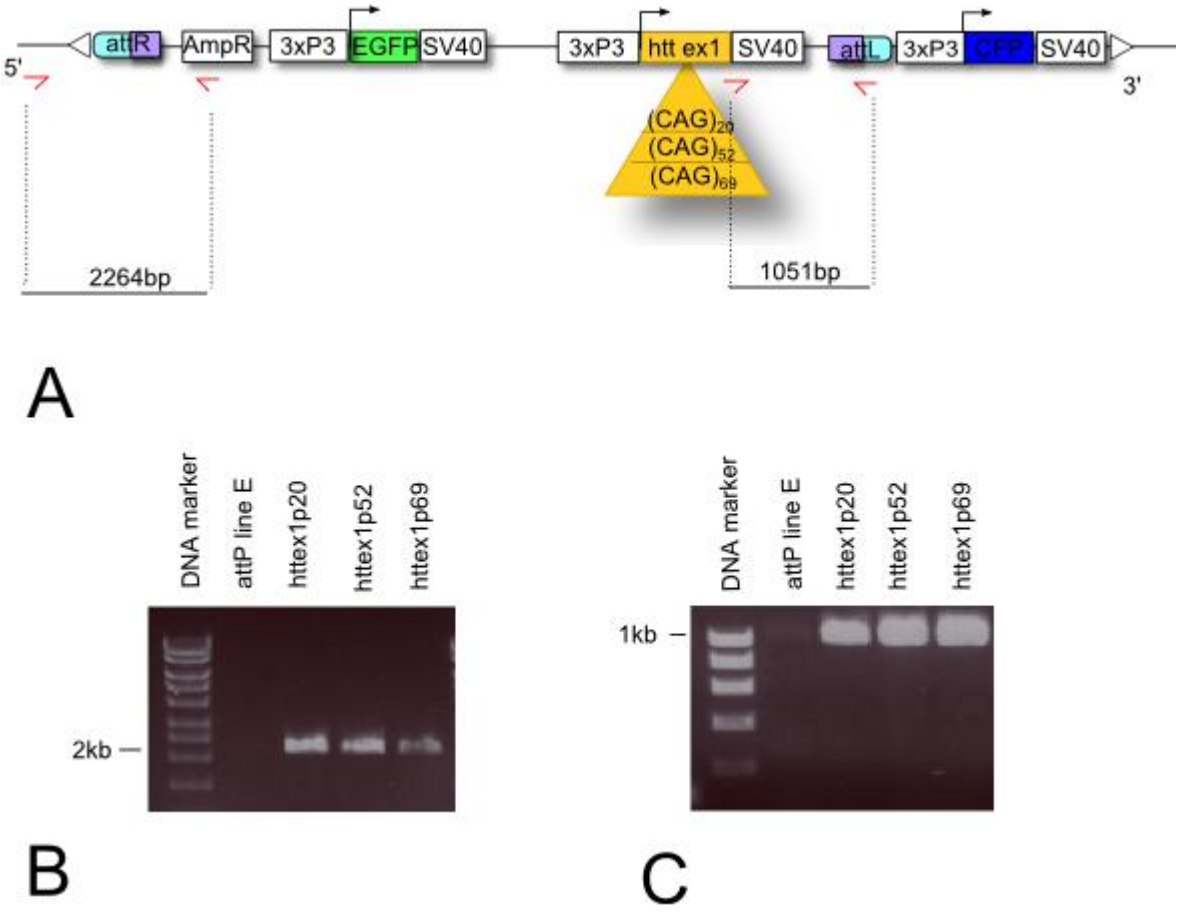


Fig.5.4: Molecular confirmation of the site-specific integration of the httex1pQ plasmids A Structure of the site-specific integration of the httex1pQ constructs into the *attP* site (resulting in *attL* and *attR* site). *3x3P*- promoter, *SV40*- terminator, EGFP and CFP- marker gene, htt ex1 -*huntingtin* exon 1 containing various lengths of CAG (Q) repeats (20, 52, 69), *attB*- insertion site of the *attP*-*attB* integration system, AmpR- *ampicillin resistance* gene. The red arrows reprsent primers that were used to amplify a fragment of 2264bp and 1051bp length (indicated with horizontal bars) specific for the httex1pQ constructs **B** Amplification of a fragment from the wildtype genome into the *ampicillin resistance* gene of the httex1pQ plasmid **C** Amplification of a fragment ranging from the last base pairs of *htt* exon 1 into the original *attP* E integration (Meredith et al., 2011).

5.4. **PolyQ repeats are unstable**

To determine the correct httex1pQ line specific polyglutamine length and to confirm that one single insertion had occurred, southern blot analysis was performed with a probe spanning the *SV40* terminator (**Figure 5.5 A, B**). Digestion of transgenic genomic DNA with the restriction enzyme *HindIII* was expected to result in three fragments spanning each a partial fragment of the 3xP3 promoter, SV40 terminator and either EGFP, CFP or httex1pQ region in the httex1pQ-transformed lines; whereas only one fragment encompassing the 3xP3 promoter, SV40 terminator and CFP was expected for the *attP* insertion line. A map of httex1pQ plasmids and httex1pQ line, indicating the restriction enzyme *Hind*III and probe used, is provided in **Figure 5.5 A**.

Digests of the httex1pQ line and injected httex1pQplasmids as control showed correct band sizes for all 3xP3-EGFP-SV40 (1207bp) or 3xP3-CFP-SV40 (1164bp) fragments. However, the fragment size of the 3xP3-httex1pQ-SV40 region varied greatly. The httex1pQ constructs from the httex1p52Q and httex1p69Q mosquito lines were shorter than the ones from the httex1p20Q line. Further, the original inserted httex1p69Q plasmid showed a smear which suggested that it contained more than one different 3xP3 httex1p-SV40 fragment. Because no change in fragment length was observed for the EGFP or CFP fragments it was believed that a change within the httex1pQ region was responsible for this variation.

Indeed genomic PCR performed over several generations confirmed that all httex1pQ lines had undergone a change in their polyQ repeat length (**Figure 5.6A-C**). The amplified regions were sequenced and revealed that after more than 16 generations, the number of repeats in the httex1p52Q and httex1p69Q line was reduced to 21, in the httex1p20Q line an expansion of 4four repeats was observed. Further, the assumption that the injected plasmid contained different polyglutamine lengths was confirmed by genomic PCR after 2 generationss which revealed 2 fragments, wherby the short fragment dominated in the following generations (**Figure 5.6A-C**). The polyQ loss within the httex1p52Q line was first observed 24 generations. Possibly due to a strong selection pressure within 8 generations over >97% of the non-homozygous httex1pQ52 population was replaced by wildtype *Anopheles gambiae* mosquitoes. The heterozygous offspring of a cross between 15 wildtype females and the remaining 1 transgenic homozygous male contained only shortened 21 polyQ fragments (**Figure 5.6 C**). The reasons for the loss of the polyQ repeats could be various. On one hand, natural selection could have favoured shorter polyglutamines. On the other hand genetic drift

by random sampling of the short gene variant could have caused the loss of the long polyQ stretches, but the fact that all offspring of the homozygous founder male contained the short allele suggests that the 21polyQ fragment persisted in this line for several generations.

Overall, similar observations have been made in a *Drosophila* HD (Marsh et al., 2000). Further polyglutamine proteins are naturally polymorphic in humans. However, it was not known, whether polyglutamine repeats are unstable in mosquitoes and how fast this change would occur in a mosquito population.

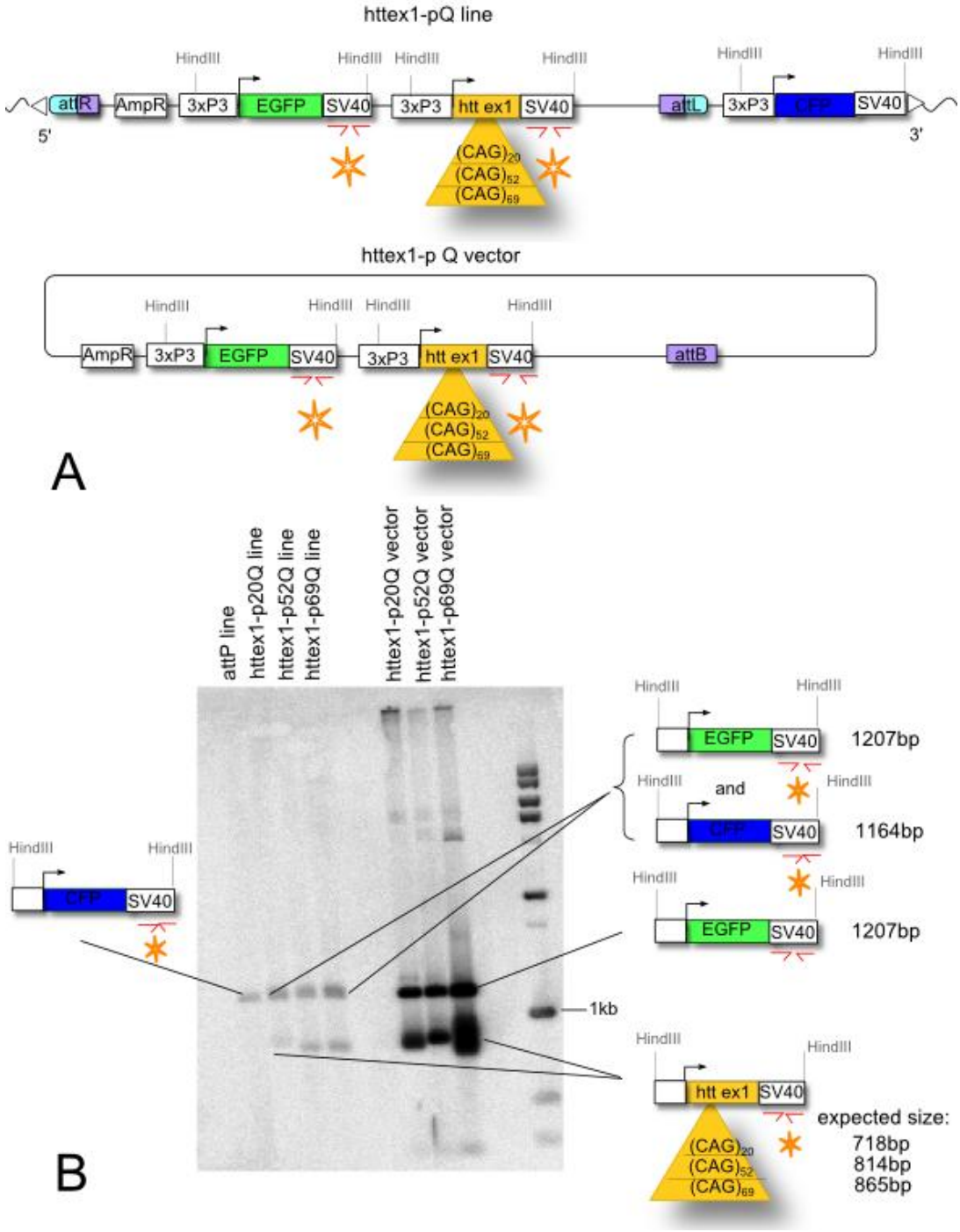


Fig. 5.5: Southern blot analysis of httex1pQ vectors and httex1pQ *An. gambiae* lines. A HindIII restriction sitesin the original httex1pQ vectors and in the httex1pQ line which are useful for fragmenting the DNA for subsequent southern blot analysis. *3x3P*- promoter, *SV40*- terminator, EGFP and CFP- marker gene, htt ex1 -*huntingtin* exon 1 containing various lengths of CAG (Q) repeats (20, 52, 69), *attB, attL, attR* sites of the *attP-attB* integration system, AmpR- *ampicillin resistance* gene. The red arrows represent primers that were used to amplify a 32P-labelled probe of 244bp covering the SV40 region which was then detected by Southern blotting (not to scale) **B** Southern blot showing the different polglutamine lengths between the original PolyQ20, 52 and 69 vector and transformed mosquitoes 16 (for httex1p20Q line), 24 (for httex1p52Q) and 19 (for httex1p69Q) generations post-insertion. As control, no httex1pQ fragment was detected in the original insertion line *attP* E.

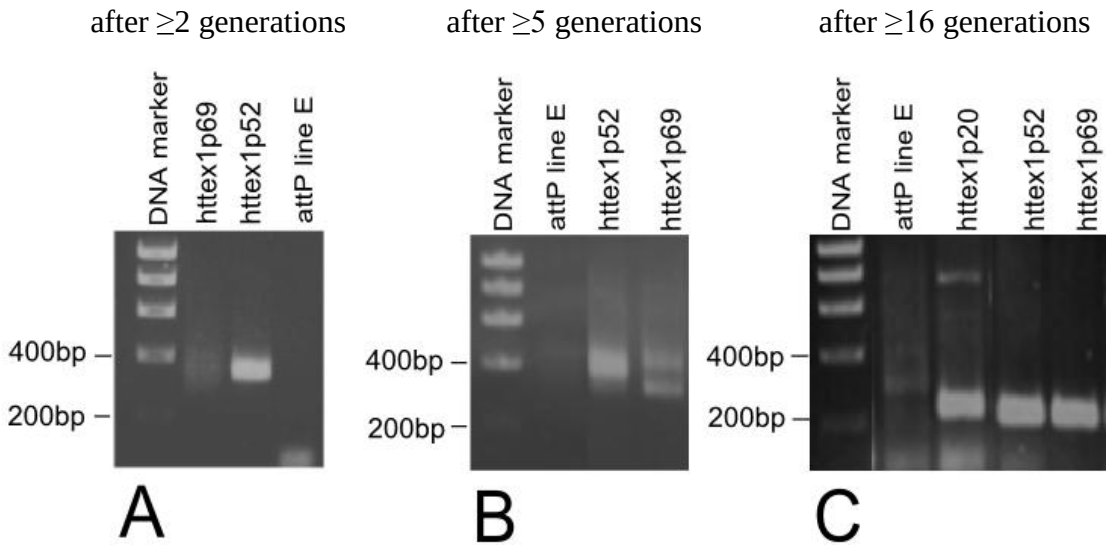


Fig. 5.6: Confirmation of polyQ instability. A The amplified polyglutamine region after 7 (for httex1p52Q line) and 2 (for httex1p69Q) generations using primers which bind the start and end of *htt* exon 1. **B** The amplified polyglutamine region after 10 (for httex1p52Q line) and 5 (for httex1p69Q) generations. **C** The amplified polyglutamine region after 16 (for httex1p20Q line), 24 (for httex1p52Q line) and 19 (for httex1p69Q line) generations.

5.5. *An. gambiae* contains endogenous polyQ proteins that might interfere with antibodies against Htt

In order to determine whether the httex1pQconstructs were functional, the respective protein had to be identified in the httex1p20Q, httex1p52Q and httex1p69 line. At this stage (after 4 generations) the httex1p69Q line had lost some of its toxic repeats, whereas httex1p52Q line still contained the putative pathogenic 52 CAG repeats. The mouse monoclonal 1C2 antibody that binds specifically polyQ stretches longer than 38 residues (MAB1574, Chemicon) and a sheep antibody (S380) that was raised against a Htt GST exon 1 fusion protein containing 51 glutamines (Scherzinger et al., 1997, Wolfgang et al., 2005) were used to detect ~15kDa in the head of httex1pQ mosquitoes. None of the used antibodies was able to detect the 15kDa protein **(Figure 5.7)**. Instead unspecific proteins of the size >30kDa were detected in the control as well as in the transformed lines. The fact that these protein bands were not only found in the transformed but also in the control line showed that these high molecular bands did not result from a HD characteristic aggregate formation of the Httex1p$Q_{>35}$ proteins. The extraction of nuclei that supposedly harbour most of the soluble toxic polyglutamine fragments did not improve the result. A blast analysis in the NCBI database revealed that in *An. gambiae* 3 proteins (AGAP006758, AGAP005135 and AGAP004734) can be found with more than 38 continues polyQ residues and a high molecular weight of 135-207kDa. Another 9 proteins contain 14-29 polyQ stretches and have a molecular weight of 15-225kDa (list can be found in the **Appendix Table 10.3**). It was therefore concluded that either Htt protein detection was hindered by binding of endogenous polyQ proteins or that the httex1pQ construct was not functional.

In order to reduce the background of most likely natural polyglutamine containing proteins, expression of these proteins in *Anopheles gambiae* Sua cells was tested but turned out to be not suitable due to low specificity for the eye- and neuron- specific *3xP3* promoter used to drive the htt exon1 expression (data not shown).

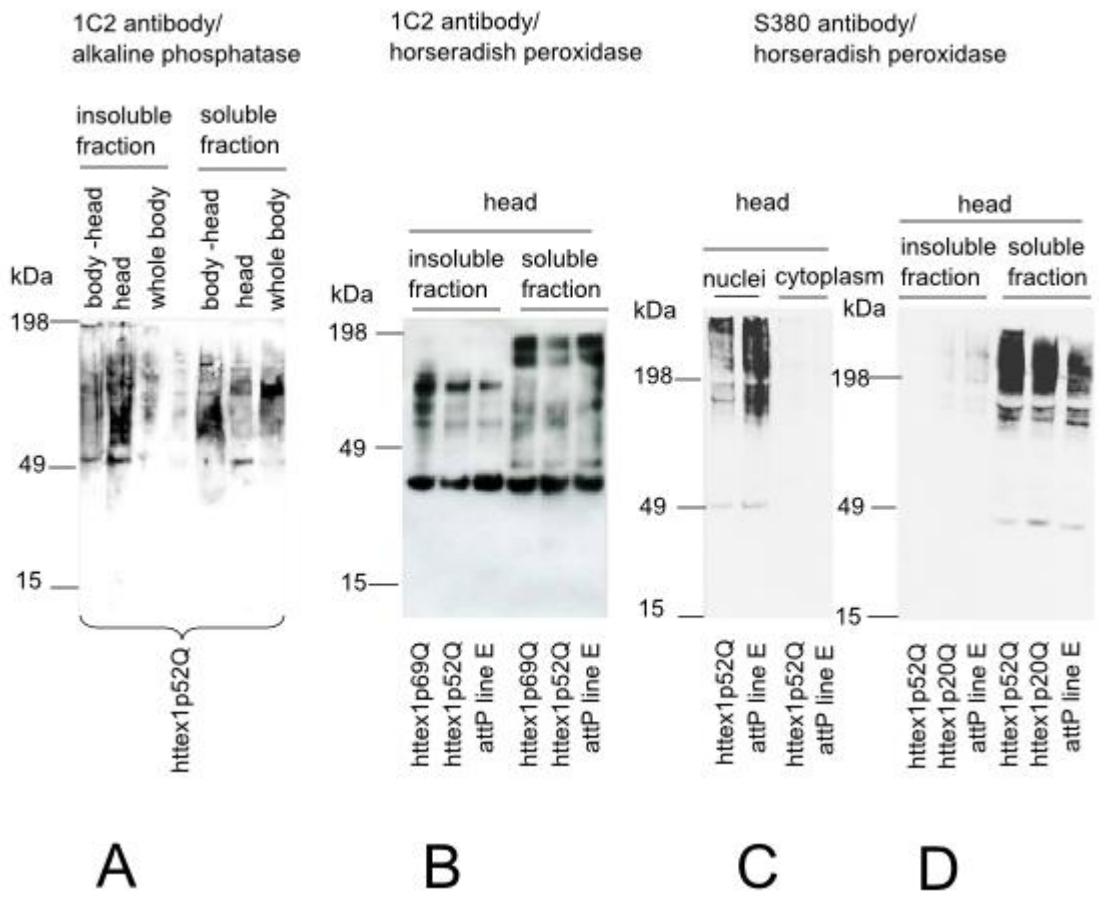


Fig. 5.7: Western blot analysis of the httex1pQ lines. A Analysis of different body parts of the E-Poly52 line with the antibody 1C2 linked to an alkaline phosphatase. **B** Protein detection with 1C2/ horseradish peroxidase in mosquito heads of the *attP* line E and httex1pQ lines **C** Detection of nuclei-extracted and non-extracted proteins from mosquito heads of the AttP line E and httex1p52Q line with horseradish peroxidise linked S830 antibody. **D** Protein detection with S380/ horseradish peroxidase in mosquito heads of httex1p20Q, httex1p52Q and *attP* line E.

5.6. Confirmation of the httex1pQ transcript expression in the transformed lines

In order to investigate whether the httex1pQ construct was functional, a qRT-PCR was performed to detect the respective transcripts. However, the repetitive nature of the polyglutamine stretches hindered the polymerase mediated amplification. Because on characteristic of the HD disease is that the CAG repeats form hairpins on the DNA and RNA level, betaine was added to solve any potential secondary structures (Henke et al., 1997). This approach was successful and facilitated the detection of the different transcripts (**Figure 5.8).** This qRT-PCR also showed that while httex1p69Q had lost a large number of polyQ repeats after >8 generations, the httex1p20Q and httex1p52Qline expressed transcripts with the expected polyQ size (185bp and 281bp respectively).

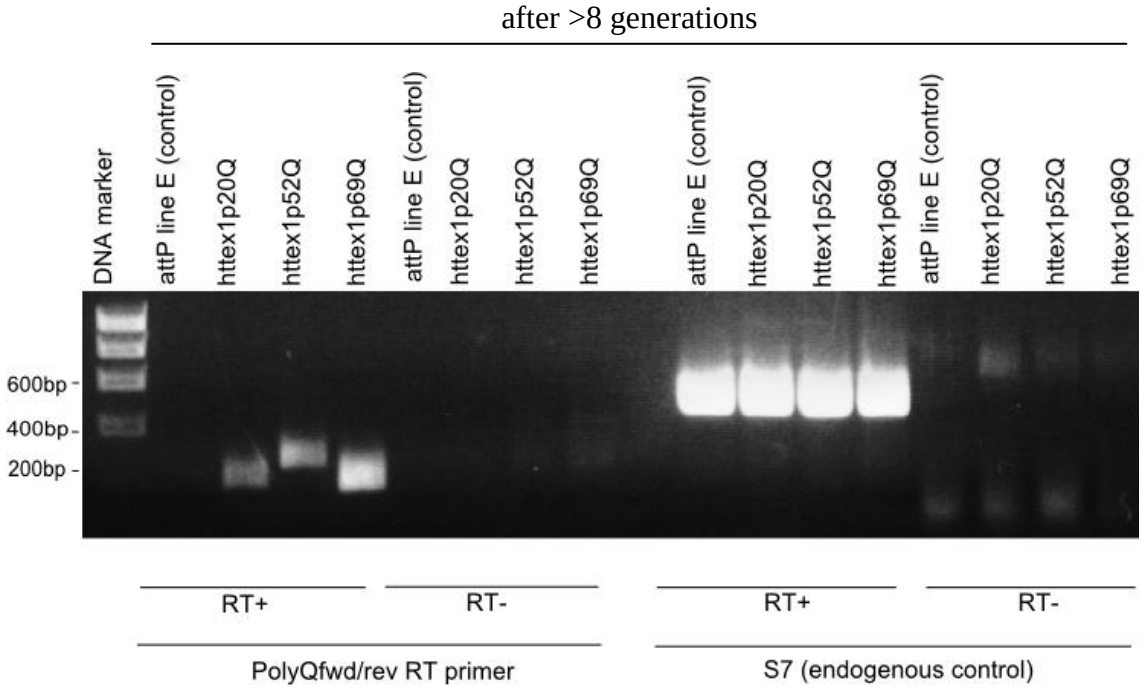


Fig. 5.8: Confirmation of the httex1pQ transcripts. The primers used for polyQ amplification were "PolyQfwd/rev RT". To ensure the presence of cDNA in all samples S7 primers were used as endogenous control. RT+ indicates the adding of reverse transcriptase which is needed for the cDNA synthesis. RT- indicates the lack of reverse transcriptase leading to no formation of cDNA, therefore any band observed in this sample results from contamination with genomic DNA. The fragment amplifying the polyglutamine stretch of the httex1p20Q and httex1p52Q region showed the correct size (185bp and 281bp respectively). At the time the samples were taken, the httex1p52Q line contained still 52Q repeats. The transcript of httex1p69Q line was shorter than expected (332bp) and contained only 21 instead of 69 CAG repeats (~200bp fragment).

5.7. Females expressing htt fragments with a pathogenic polyQ$_{52}$ stretch have a reduced median life span

In order to investigate the potential toxicity of expanded polyglutamine stretches, the daily survival of adult males and females of the different httex1pQ lines was recorded after >8 generations. Unfortunately the httex1p69Q line had lost 45 CAG repeats at this stage and was therefore expected to show a similar survival as the httex1p20Q and *attP* line E. The resulting survival curves were analysed by Kaplan Meier analysis (SPSS statistics 17.0) and showed no significant difference between the httex1pQ expressing males. The shape of the survival curve of the attP line differed from all other lines, 52% of the males of the attP line died within a very short time (6 days) suggesting a non-natural death **Figure 5.9** and **Table 5.2.** While no polyQ dependent death was observed in males, Httex1p52Q females which expressed a Httex1 fragment with 52 CAG repeats had a 18% shorter median life span compared to all other transgenic lines (log-rank test, χ^2 = 5.18, P =0.02). The survival between the *attP* line E, httex1p20Q and httex1p69Q line did not differ. This finding supported the hypothesis that polyglutamine stretches larger than 21 repeats are toxic in mosquitoes. Given that at the time of the life span assay the line was not purified to homozygousity, the observed life span could probably be an under estimate. However, in humans, HD is dominantly inherited and the difference in severity between individuals that are homozygous or heterozygous for polyglutamine expansions is controversial ((Wexler et al., 1985, Wexler et al., 1987, Squitieri et al., 2003).

Unfortunately these experiments could not be repeated due to the loss of polyglutamine repeats in the httex1p52Q line, leaving only non- toxic 21 polyQ repeats. However, this result was promising for further approaches using more stable polyQ constructs.

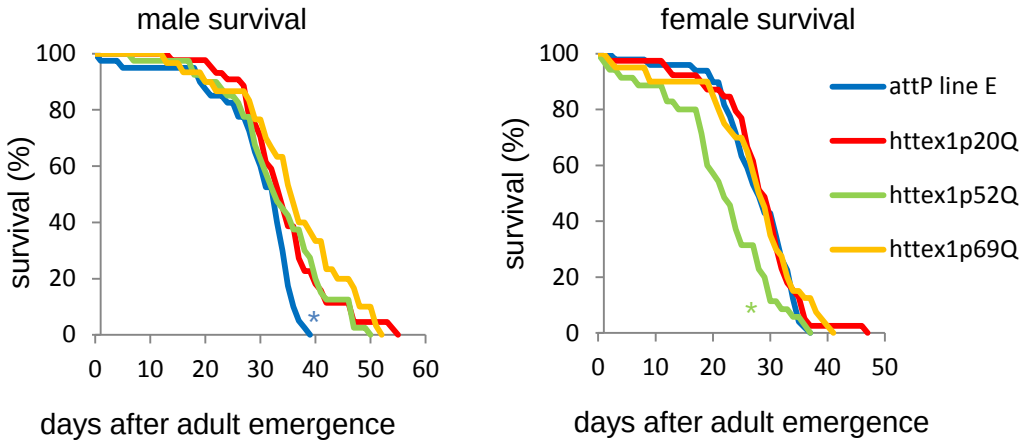


Fig. 5.9: Females expressing httex1p52Q have a reduced median life span. Displayed is the percentage of male and female mosquitoes alive after adult emergence over a period of ~50 days (log-rank test, * p< 0.05). The survival of mosquitoes of the httex1p69Q line was measured after they had lost the PolyQ repeats from 69 to ~21 repeats.

Table 5.2: Survival of male and female httex1pQ *An. gambiae* mosquitoes.

gender	transgenic line	no. of polyQs	no. of mosquitoes	median	maximum	p- value (Log-rank test comparing the survival of the different transgenic lines with httex1p20Q)
male	attP line E	0	38	33	38	0.01
	httex1p20Q	20	42	34	47	
	httex1p52Q	52	40	35	48	0.10
	httex1p69Q	21*	27	35	52	0.25
female	attP line E	0	48	28	35	0.56
	httex1p20Q	20	34	28	46	
	httex1p52Q	52	38	23	36	0.02
	httex1p69Q	21*	40	28	41	0.42

Mean and maximum survival times in days were calculated from survival curves using Kaplan-Meier analysis. P-values were calculated using log rank statistics and compared the "non-pathogenic" httex1p20Q line with the *attP* line and mosquitoes expressing httex1p52Q, httex1p69Q (21Q). P<0.05 indicates a significant reduced life span of this transgenic line compared to the httex1p20Q control. * The survival of mosquitoes of the httex1p69Q line was measured after they had lost the PolyQ repeats from 69 to ~21 repeats.

5.8. Development of transformation vectors that contain stable alternating CAG/CAA residues fused to EGFP

In light of the previously reported technical problems relating to the instability of CAG repeat stretches encoding polglutamines and the inability to detect proteins in a Western blot using available Htt antibodies another strategy was to express a huntingtin N-terminal cDNA fragment that contained a Kozak sequence to optimise translational efficiency and the first 17 amino acids of the Htt exon 1 and alternating CAG/CAA triplet repeats (CAA CAG CAG CAA CAG CAA)n encoding either 25 glutamine (Q) residues (normal in HD) or extended 97 glutamine residues (elongated beyond pathological range) (Kazantsev et al., 1999). These htt peptides had been shown to be highly stable during bacteria propagation and formed pathogenic polyQlength dependent aggregates in mammalian cos-7cells, yeast, mice and recently in the fruit fly (Meriin et al., 2002, Muchowski et al., 2008, Zhang et al., 2010, Weiss et al., 2011, Lecerf et al., 2001). Further, a 230-amino acid EGFP tag at the C-terminus of the construct allowed easy monitoring of aggregate formation in cells in these experiments (Kazantsev et al., 1999). Since expression of polyQEGFP fusion proteins could not be assured to work as a transformation vector, another RFP marker gene with known expression pattern was cloned into the final httN-QEGFP vector (**Figure 5.10 A, B**).

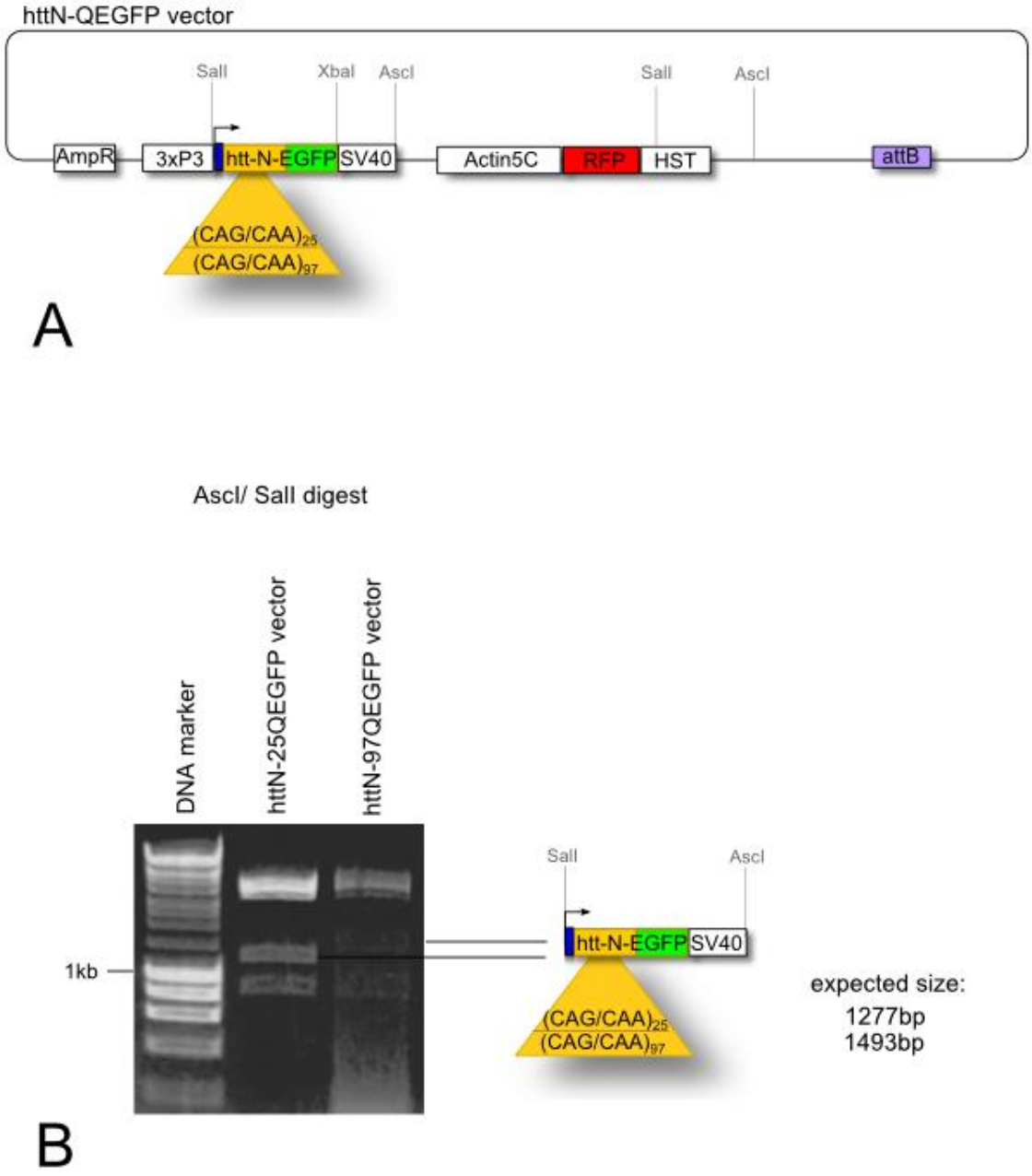


Fig. 5.10: Generation of the htt-NQEGFP vector. A httN-QEGFP vectors containing alternating CAG/CAA triplet repeats encoding 25 glutamine residues (normal in HD) and extended 97 glutamine residues (elongated beyond pathological range) which are part of a huntingtin N-terminal cDNA fragment, including the Kozak box (in blue) and the first 17 aa. Further, a 230-aa EGFP tag (in green) at the C-terminus of the construct allows easy monitoring of aggregate formation (Kazantsev et al., 1999). The restriction sites used for cloning are included. *3x3P*- promoter, *SV40*- terminator, Actin5C-promoter, HST- terminator, EGFP and RFP- marker gene, attB- part of the attB-attP integration system **B** SalI/AscI digest of the 2 httN-QEGFP vectors results in 4 fragments of which one concists of 1277bp (httN-25QEGFP) and 1493bp (httN-97QEGFP) due to the different CAG/CAA length.

5.9. Generation of httN-25QEGFP and httN-97QEGFP expressing flies

Given the previous problems it was decided to test the validity of these constructs in the fly model, for which there are well established protocols for qualifying neurodegeneration or behavioural changes. Firstly, the stability and expression pattern of httN-25QEGFP and httN-97QEGFP fusion proteins was validated in a well established neurodegenerative disease model, *D. melanogaster*. The generated plasmids httN-25QEGFP and httN-97QEGFP were injected into the *attp* fly line *y1 w67c23; P{CaryP}attP2* (Bloomington stock 8622). The outcome of the injection results can be seen in Table 3.5. 2 of 19 fertile individual crosses with the white eyed w^{1118} line at a ratio of 1:4 resulted in 94 transgenic httN-25QEGFP G_1 progeny. Only 1 cross of a G_0 injected male with a virgin w^{1118} female gave 48 transgenic httN-97QEGFP progeny (**Table 5.3**). Although the molecular nature of the integration was the same lines were generated from three different G1 transgenic httN-25QEGFP and httN-97QEGFP flies to control for any founder effect that might confound a phenotype related to the transgenic inserts. Subsequently all virgin G_1 httN-QEGFP flies were crossed individually in a ratio of 1:4 to the double balanced line w^{1118}*; TM2, Ubx/TM6C,Sb* (*stubble*). The resulting 25% of the G_2 progeny which had visble GFP fluorescent eyes (httNQEGFP) and stubbly bristles were then crossed with each other giving homozygous httN-QEGFP G_3 progeny with normal bristles.

Table 5.3: Outcome of httNQEGFP plasmid injections into *D. melanogaster* embryos.

injected plasmid	injected embryos	hatched adults	crossing scheme	fluorescent/ total G1	transient G0founder	established lines
httN-25QEGFP	200	28(14%)	outcross to w1118	94/2081 (4.5%)	1 male	httN25QEGFP_1 a and b
					1 female	httN-25QEGFP_2
httN-97QEGFP	270	19 (7%)	outcross to w1118	48/1462 (3%)	1 male	httN-97QEGFP a, b and c

5.10. **Confirmation of the site-specific integration of httN-QEGFP constructs in transgenic flies**

The site-specific integration into the *attP* site of the *y1 w67c23; P{CaryP}attP2* line was confirmed by genomic PCR using a primer binding in the *ampR* gene of the httN-QEGFP constructs and another binding in a region specific for the orginal *attP* insertion (**Figure 5.11 A,B**).

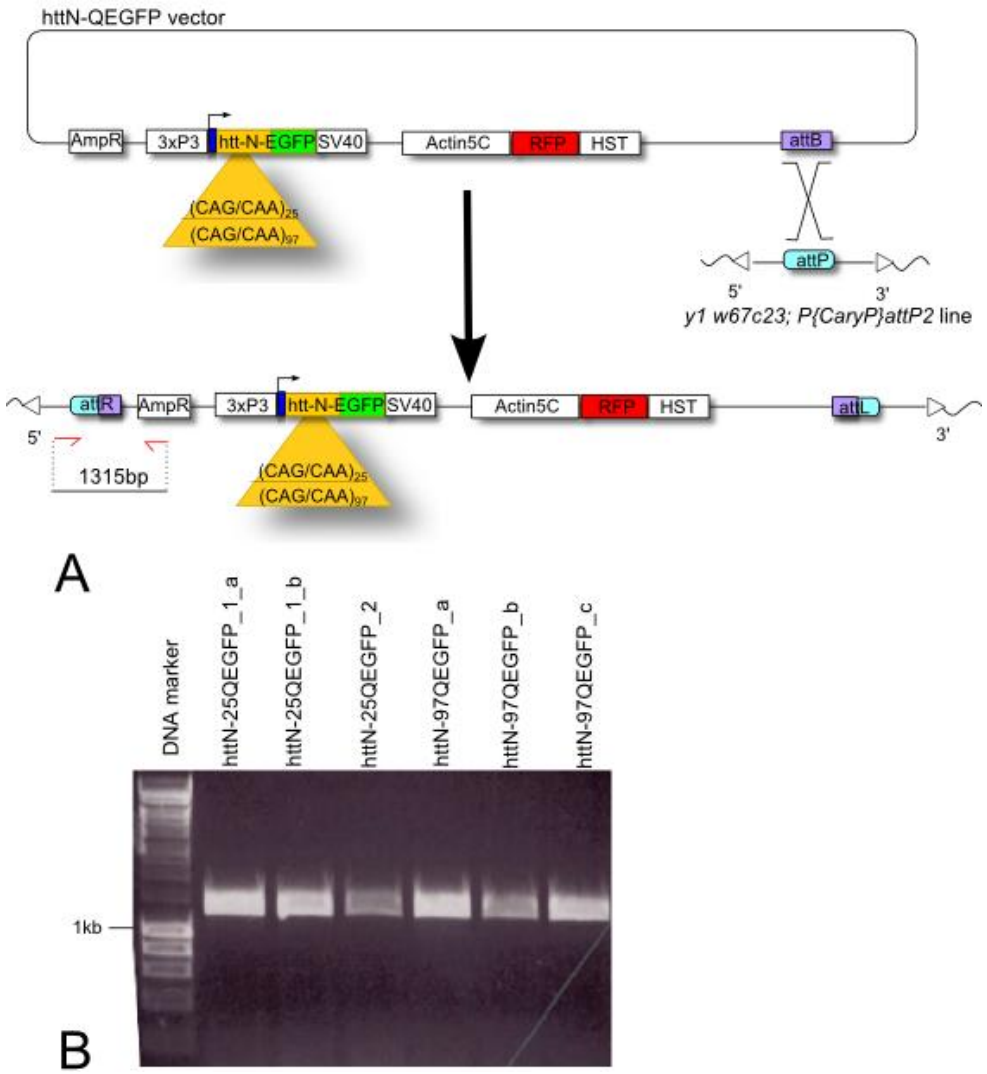


Fig. 5.11: Site-specific integration of httNQEGFP into *D. melanogaster*. **A** Site-specific integration of the httNQEGFP constructs into the *attP* site (resulting in *attL* and *attR* site) of the fly line *y1 w67c23; P{CaryP}attP2*. *3x3P*- promoter, *SV40*- terminator, *EGFP* and *RFP*- marker gene, httN-EGFP- huntingtin N-terminal cDNA fragment containing $(CAG/CAA)_{25}$ or $(CAG/CAA)_{97}$ repeats which is fused to EGFP, *attB*- insertion site of the *attP-attB* integration system, AmpR- *ampicillin resistance* gene. The red arrows represent primers that were used to amplify a fragment of 1315bp length (indicated with horizontal bar) in a genomic PCR **B** Genomic PCR of the httN-QEGFP fly lines showing a fragment amplified from the original inserted attP integration construct into the *ampicillin resistance* gene of the httNQEGFP plasmid.

5.11. **Alternating (CAG/CAA)$_n$ residues are stably inherited in httN-QEGFP expressing flies**

Using genomic PCR two polyQ length dependent fragments of 523bp and 739bp ranging from the N-terminal end of *htt* into the *EGFP* region were amplified from DNA of the httN-25QEGFP and httN-97QEGFP fly lines. Importantly, over 17 generations the length of these amplicons remained stable in all fly lines (**Figure 5.12 A, B**).

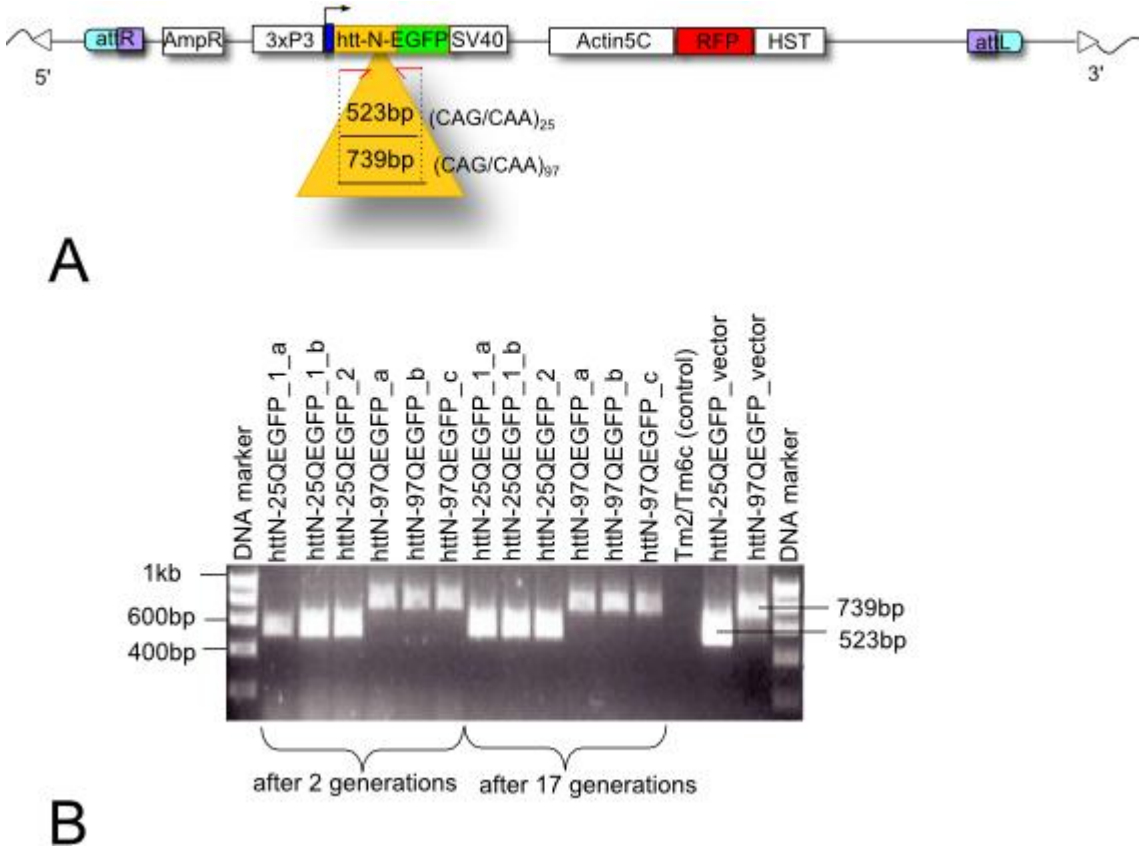


Fig. 5.12: CAG/CAA repeats are stable. A Figure shows the transgenic elements of the httNQEGFP line. *3x3P*- promoter, *SV40*- terminator, *EGFP* and *RFP*- marker gene, httN-EGFP- huntingtin N-terminal cDNA fragment containing $(CAG/CAA)_{25}$ or $(CAG/CAA)_{97}$ repeats which is fused to EGFP, *attB*- insertion site of the *attP-attB* integration system, AmpR- *ampicillin resistance* gene. The red arrows represent the primers used to amplify a fragment including the 25Q (CAG/CAA) (523bp) or 97Q (CAG/CAA) repeats of the original httN25QEGFP and httN97QEGFP line. **B** Genomic PCR showing the 2 polyQ size specific fragments amplified in the httNQEGFP vectors as well as in the transgenic polyQ25 or 97 containing fly lines after 2 and 17 generations. The Tm2/Tm6c balancer line was used as negative control.

5.12. **HttN-97QEGFP expression leads to aggregate formation in the fly brain**

The eye- specific expression of the httN-QEGFP fusion protein allowed simple detection and of the 2 different polyglutamine containing proteins under a fluorescence light microscope. In order to examine the Htt expression pattern in the fly brain, female adult brains were immunostained with an antibody against Protein kinase C (anti-PKC) which has been used to stain the neuropil (optic ganglia) (H. Apitz, personal communication) and visualised by confocal microscopy. While the *3xP3* eye-specific promoter expressed the "non-pathogenic" httN25QEGFP protein in a regular fashion in the lamina and medulla of the optic lobe, httN97EGFP formed irregular aggregates in the whole adult brain (**Figure 5.13 A,B,C**). Interestingly, in contrast to the httN25QEGFP protein expression, httN97QEGFP expression in the medulla was completely absent (**Figure 5.13 D, E, F**). In general this optic ganglion is innervated by long axonal projections from the inner photoreceptors R7 and R8 in the retina, which mediate colour vision (Jackowska et al., 2007). All other photoreceptors terminate their projections in the lamina.

In order to determine whether the absence of aggregate formation in the medulla because of degeneration of these photoreceptor axons, pupal fly brains expressing 25 or 97 polyglutamines were dissected and stained with the monoclonal antibody against chaoptin (mAb24B10) which stains exclusively larval and pupal photoreceptor cells in the retina and their axonal projections to the 2 optic ganglia: the lamina and medulla (Van Vactor et al., 1988, Gontang et al., 2011). HttN25QEGFP and Anti-Chaoptin stained the photoreceptor cells and its axonal projections (**Figure 5.14 A**). A faint stain of the anti- chaoptin antibody was found in the medulla of httN97QEGFP expressing flies, which was less visible deu the missing EGFP background suggested that no degeneration of the R7/R8 axons had occurred (**Figure 5.14 B**). The irregular GFP pattern in the retina could be explained by aggregate formation in the nucleus of the photoreceptor cells, which is characteristic for mutated htt (deRooij et al., 1996) (**Figure 5.14 C,D)**. However, other aggregates in the lamina and surrounding tissue (possibly glial cells) suggest that polyQ aggregation is not limited to the nucleus.

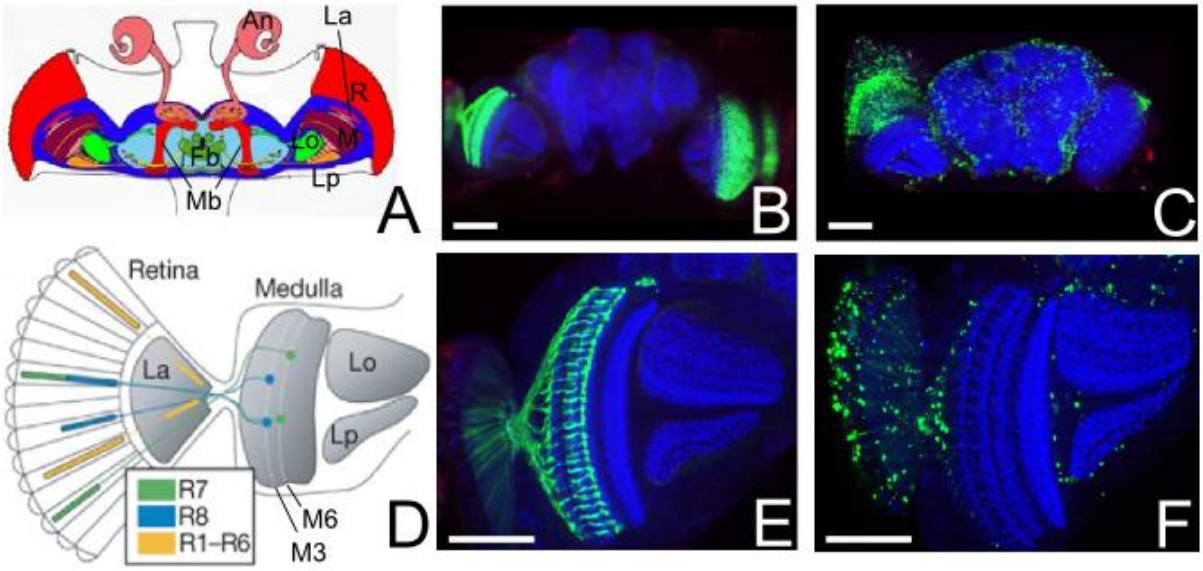


Fig. 5.13: HttN-QEGFP expression in the adult fly brain. A Schematic view of the *D. melanogaster* brain. An- Antenna, Fb- Fan shaped body, La-Lamina, Lo-Lobula, Lp-lobula plate, M-medulla, R- Retina, (http://filab.biologie.uni-freiburg.de/Atlas/text/) **B** Dissected and immunostained whole brain of adult httN-25QEGFP female fly showing the httN25-enhanced green flouresecent protein (GFP) (in green) in the R cells and the neuropil stained with Anti-PKC (in blue) **C** Dissected and immunostained whole brain of adult httN-97QEGFP female fly showing the httN97QEGFP fusion protein (in green) in the R- cells and surrounding glial cells and the neuropil staned with Anti-PKC (in blue) **D** Schematic representation of *Drosophila* R cell projections from the adult retina into the lamina (La, R1–R6) and medulla (R8/R7) optic ganglia; more central optic ganglia are the lobula (Lo) and the lobula plate (Lp) (Petrovic and Hummel, 2008). **E** Projection of httN-25QEGFP line R7 and R8 axons marked with httN25QEGFP, into the M6 and M3 synaptic layer, respectively. The neuropil is stained with Anti-PKC (in blue) **F** Disruption of the projection of httN-97QEGFP R7 and R8 axons marked with httN97QEGFP into the medulla. Instead, aggregate formation of 97QEGFP can be observed in the retina, lamina as well as the surface- and neuropil glial cells. The neuropil is stained with Anti-PKC (in blue).

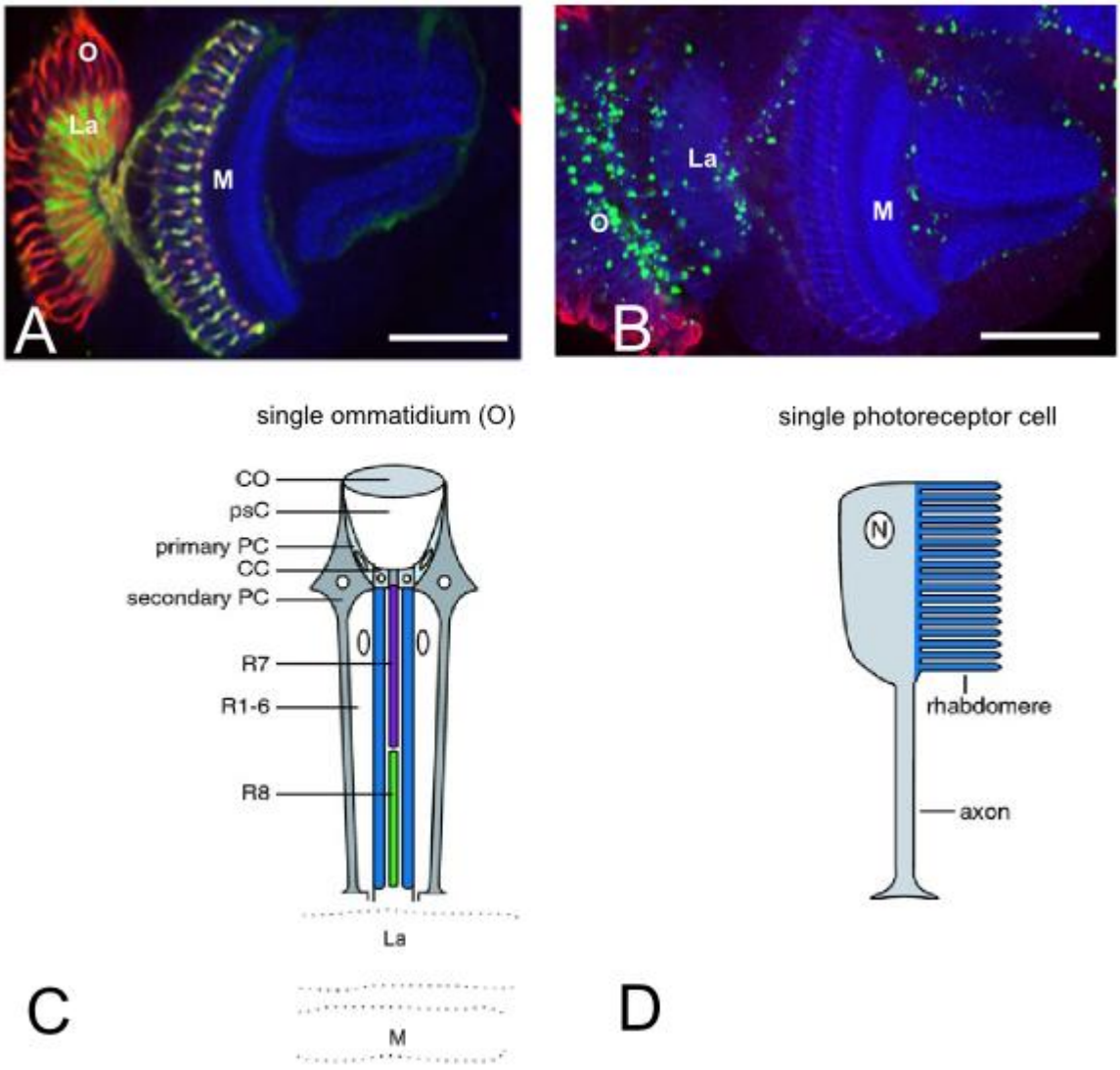


Fig. 5.14: HttN-QEGFP expression in the photoreceptor stained pupal optic lobe. A Optic lobe of a pupa fly expressing httN25QEGFP in the photoreceptor cells. The photoreceptor cells (O-ommatidium) and their axonal projections into the lamina (La) (R1-R6) and medulla (M) were stained with the Anti- Chaoptin antibody. Anti-PKC marked the neuropil. Scale bar 100µm **B** Optic lobe of a pupa fly forming httN97QEGFP aggregates in the lower part of the ommatidia (O) and lamina (La). The photoreceptor cells (O-ommatidium) were stained with Anti- Chaoptin. Scale bar 100µm **C** Single ommatidium at an angle 90° from the surface of the compound eye: Co- cornea, primary PC- primary pigment cell, psC- pseudocone, secondary PC- secondary pigment cell, R1–6- photoreceptor cells 1–6, R7- photoreceptor cell 7, R8- photoreceptor cell 8 (Wang and Montell, 2007) **D** Longitudinal view through a photoreceptor cell. The rhabdomere, axon, and nucleus (N) are indicated (Wang and Montell, 2007).

5.13. **HttN-97QEGFP expression in flies causes a locomotory defect and reduced life span**

To investigate whether the eye-specific expression of the pathogenic 97 polyglutamines had an effect on the fly life span, the median life span of male and virgin female adults of the three established httN-97QEGFP and httN25Q-control lines was compared. A detailed pairwise comparison can be found in the appendix (**Appendix Table 10.4, 10.5 and 10.6**). Overall, the median life span of httN97QEGFP male flies was with 66 days almost 10% shorter than that of httN25QEGFP control males (**Table 4.4, Figure 5.15A)**. Moreover, in females a 32% reduction in life span was observed in those expressing httN97QEGFP compared to the htt25QEGFP controls. A lower larval density of the httN97QEGFP_c line could have accounted for the relatively high survival of the adult males and females.

Because Huntington's- disease is an age-related motor neuron disease, it was determined whether the expression of pathogenic httN97QEGFP had an effect on adult locomotor function using a well-established adult climbing assay that had been used extensively to characterise neurodegenerative diseases in *Drosophila* models (Rival et al., 2004). Females as well as males expressing the pathogenic httN97QEGFP fusion protein showed an age-dependent decline of their climbing ability (**Figure 5.15B**). Compared to httN25QEGFP males, a significant reduction in climbing ability was observed from day 49 onwards for httN97QEGFP expressing males. The age-specific decline in the climbing behaviour was similar between female flies of both groups. Overall these two experiments showed that the htt-N97QEGFP fusion proteins do not only form aggregates in the fly brain but also have a pathogenic effect by causing a progressive adult locomotory defect and decline in the life span of male and female flies.

Table 4.4: Survival analysis adult male and female httN25QEGFP and httN97QEGFP expressing *D. melanogaster*

gender	no. of PolyQs	n	mean (in days)	median (in days)	maximum (in days)	p- value (log-rank test)
male	25	107	64	73	101	
	97	92	57	66	94	0.02
female	25	131	91	108	134	
	97	98	74	73	102	<0.0001

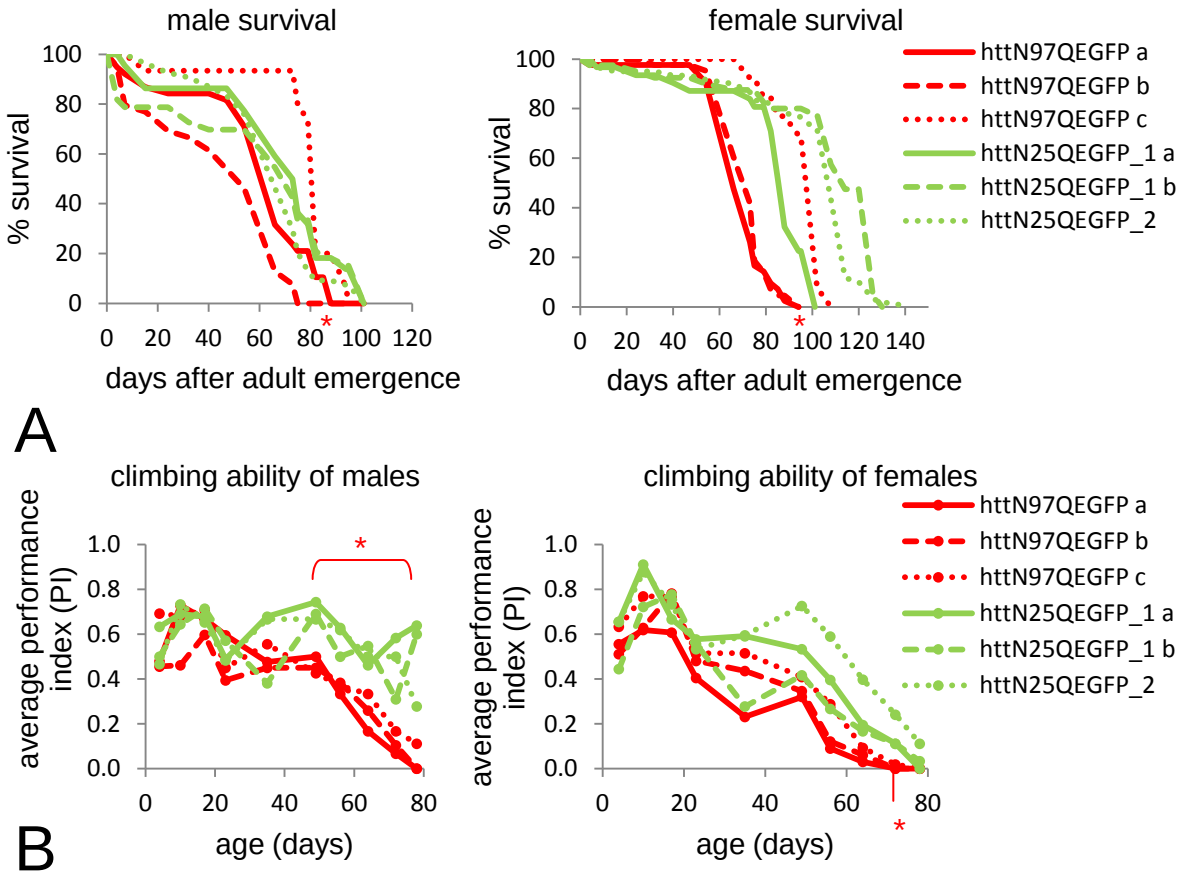


Fig. 5.15: HttN97QEGFP flies have a reduced life span. A Survival curves of three httN25QEGFP and httN97QEGFP *D. melanogaster* populations showing the percentage of male and virgin female flies alive after adult emergence over a period of ~140 days. Overall male and female httN97QEGFP fly populations lived significantly shorter than the httN25QEGFP controls (Log-rank test, three strains, males $p<0.05$, females $p<0.0001$). **B** Climbing ability of males and females expressing httN25QEGFP or httN97QEGFP. 15 flies were tapped to the bottom and after 45sec. the number of flies (n) on the top and bottom of the vial was determined. This procedure was repeated three times. The average was used to calculate the performance index (PI) = 0.5 x (n total +n top -n bottom)/ n total). (unpaired t-test, * $p<0.05$).

5.14. **Generation of transgenic *An. gambiae* mosquitoes expressing httN-QEGFP**

After httN97QEGFP proteins were shown to be pathogenic and stably inherited over several generations in flies, the generated vectors httN25QEGFP and httN97QEGFP were injected firstly into the *attP line* E and then in the newly established marker-less X1 attP insertion line (Meredith et al., 2011). The capped helper φC31 Integrase RNA was replaced by a newly available plasmid which carried the *vasa* promoter expressing the Integrase specifically in the germline (Papathanos et al., 2009). The overview of the injection results is shown in **Table 5.5**. Transgenics were identified by their *3xP3* mediated EGFP (directly fused to httNQ) and *Actin*-RFP characteristic expression (**Figure 5.16**). While only 1 transgenic httN25QEGFP expressing female was derived from one female G_0 founder, 5 transgenic httN97QEGFP mosquitoes were obtained from at least 2 injected females and 1 injected male. Only 1 transgenic G1 female per construct was crossed with ~30 wild type males to obtain a heterozygous httNQEGFP line.

Table 5.5: Overview of the injections of different httN-QEGFP plasmids into *attP* line E and marker less *X1* line *Anopheles gambiae* embryos.

Injected *attP* line	injected plasmid	injected embryos	hatched larvae	transient adults	crossing scheme	fluorescent/ total G1	transient G0 founder
attP line E	httN25Q-EGFP	807	76 (9%)	5 (0.6%)	Wt outcross	0/1558	0
attP line E	httN97Q-EGFP	1385	173 (13%)	17 (1.2%)	Wt outcross	0/1969	0
X1 line	httN25Q-EGFP	990	48 (5%)	6 (2.5%)	Wt outcross	1/2235 (0.04%)	1 female
X1 line	httN97Q-EGFP	2309	119 (5%)	17 (0.7%)	Wt outcross	5/11591 (0.04%)	min. 2 females and 1 male

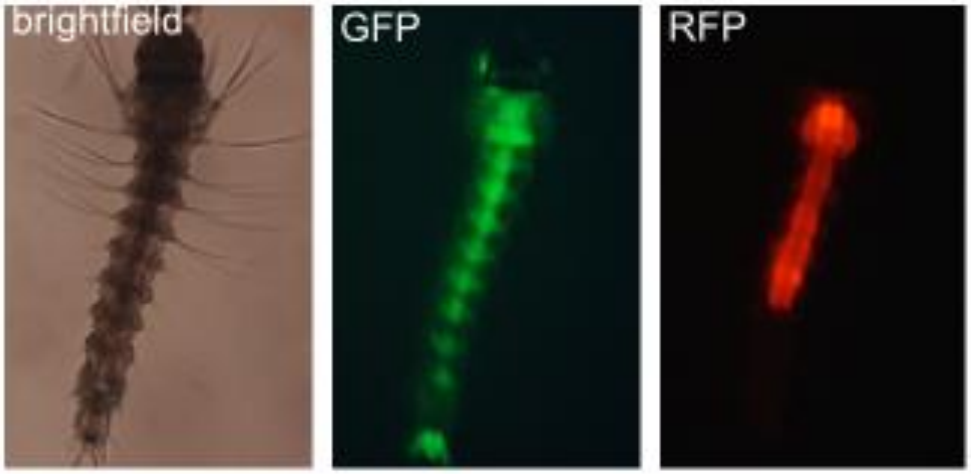


Fig. 5.16: Expression of httN25QEGFP construct in a transgenic *An. gambiae* larva. Transgenic httN25QEGFP larva which expresses the *3-P3*-httN25QEGFP fusion protein and *Actin*-RFP.

5.15. **A robust novel technique to generate homozygous mosquitoes**

Depending on the promoter, in some *An. gambiae* lines it is possible to distinguish homozygous from heterozygous individuals by the intensity of fluorescence under the microscope, but often this method is not 100% accurate. High throughput sorting by Complex Object Parametric Analyzer and Sorter (COPAS) has been used to sort transgenic *Anopheles* and *Drosophila* larvae and can distinguish between hetero and homozygotes based on empirically-optimized gating of transgene fluorescence levels (Catteruccia et al., 2005, Rasgon, 2009). However, this system is expensive and thus not widely applied.

Here, a new method of sorting homozygous transgenic mosquitoes was utilised (Naujoks, personal communication). This approach required two transgenic lines which contain different fluorescent marker insertions in the same attP Locus. Alternatively, instead of different fluorescent markers, different promoters can be used. Crossing of these two lines resulted in progeny which expressed both marker genes heterozygously. These were then selected and intercrossed with each other. The following generation 50% of the progeny carried either one or the other transgenic marker. These mosquitoes were homozygous for the respective transgene and were then intercrossed to maintain a homozygous mosquito population. A summary of the crossing scheme of the httNQEGFP lines is displayed in **Figure 5.17**.

In order to avoid any confounding effects on the httNQEGFP survival from the crossing with the VFS-1 (*3xP3*-RFP) line, the wildtype and X1 line controls were also outcrossed to the VFS-1 (*3xP3*-RFP) line and the resulting heterozygous progeny was crossed with each other to regain homozygous wildtype and X1 line mosquitoes.

Overall, different “mosquito lines” expressing various fluorescent marker genes under different promoters could be established for different *attP* integrations and used for the generation of homozygous lines.

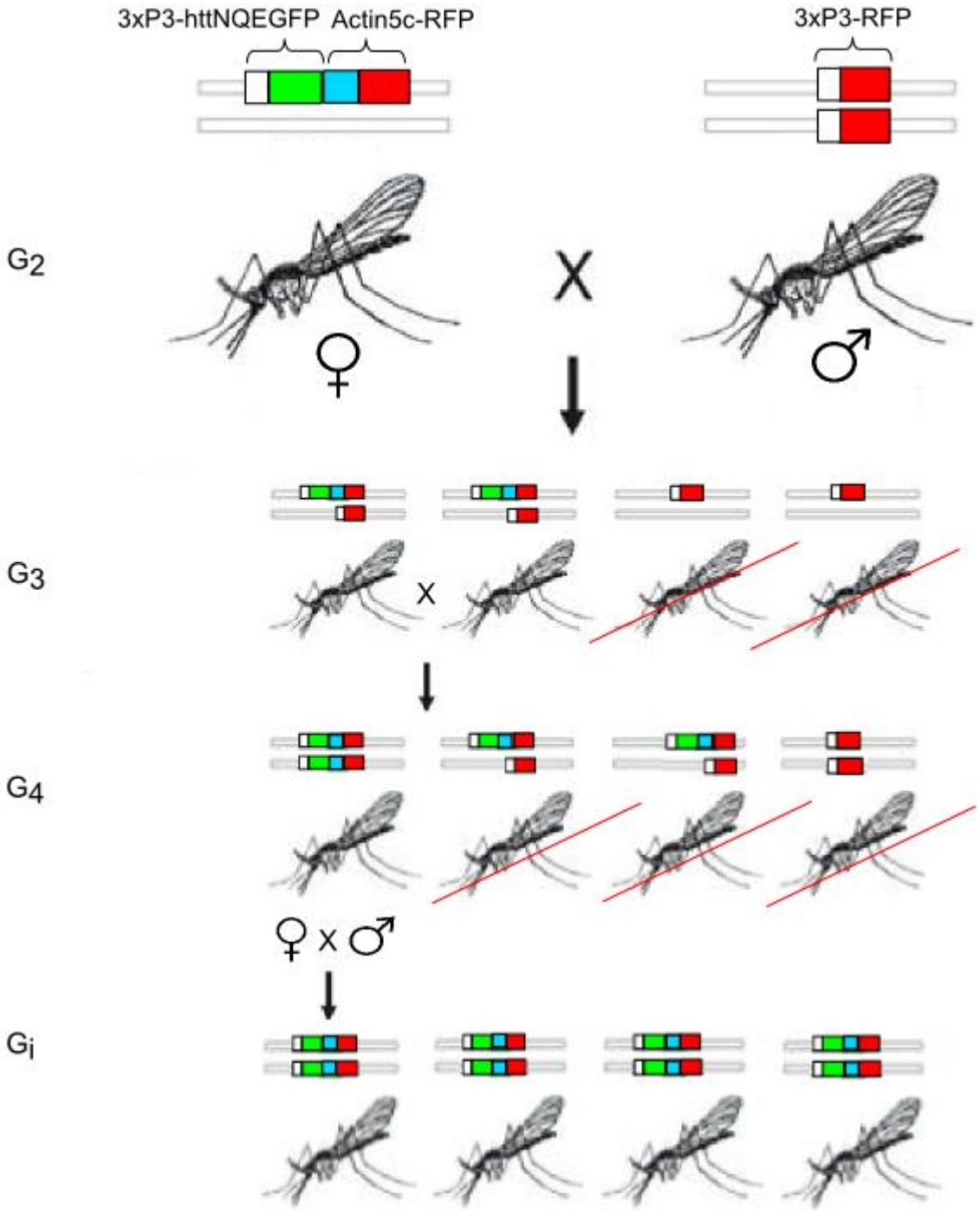


Fig. 5.17: Crossing scheme to establish the homozygous httN-25QEGFP and httN-97QEGFP lines. Heterozygous female G_2 httQEGFP mosquitoes were crossed to males of the homozygous VFS-1 line, which contained a visible 3xP3 RFP marker at the exact location as the httN-QEGFP-ActinRFP insertion, due to integration into the same *attP* insertion line X1. The heterozygous httN-QEGFP/VFS-1(3xP3RFP) G_3 progeny (50% of the offspring) was then crossed with each other resulting in 25% homozygous httN-QEGFP, 25% homozygous VFS-1(3xP3RFP) and 50% heterozygous httN-QEGFP/VFS-1(3xP3) mosquitoes. The homozygous httN-QEGFP individuals were crossed with each other in all following generations (G_i).

5.16. **Confirmation of the site-specific integration and correct polyglutamine size of the httNQEGFP mosquito lines**

Genomic PCR was performed on G1 transgenic httN25QEGFP and httN97QEGFP mosquitoes using primers which bind the wildtype genome and the *AmpR* gene of the httNQEGFP region. While no fragment was detected in the negative control (X1), in both httNQEGFP lines a fragment of 1981bp was amplified indicating a site-specific integration of the construct into that locus (**Figure 5.18 A, B**). Importantly amplicons of 523 and 739 bp from the polyQ25 and polyQ97 region were consistant in their size compared to their respective transgenic flies having retained the full complement of polyglutamine repeats.

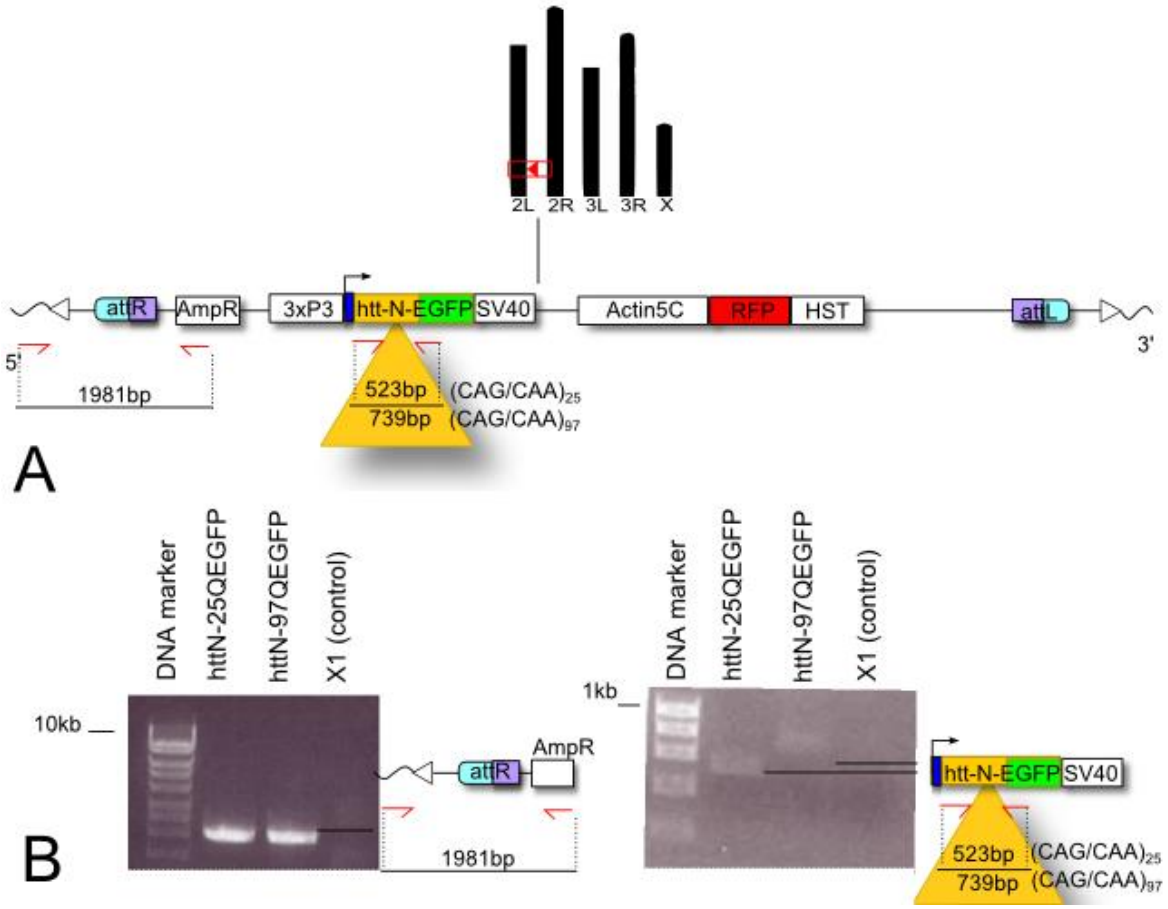


Fig. 5.18: Confirmation of the site-specific integration of httNQEGFP. A Site-specific integration of the httNQEGFP constructs into the *attP* site (resulting in *attL* and *attR* site) of the *An. gambiae X1* line. *Actin5c* and *3x3P*- promoter, *SV40*- terminator, *RFP*- marker gene, httN-EGFP- huntingtin N-terminal cDNA fragment containing $(CAG/CAA)_{25}$ or $(CAG/CAA)_{97}$ repeats which is fused to EGFP, *AmpR- ampicillin resistance* gene. The red arrows represent primers that were used to amplify a site-specific fragment of 1981bp length (indicated with horizontal bar) and polyglutamine repeat- specific fragment of 525bp and 741bp by genomic PCR **B** Genomic PCR of the httN-QEGFP mosquito lines showing site-specific fragments of 1981bp and polyQ fragments of 523bp and 739bp.

5.17. **HttN-97QEGFP aggregate formation in the mosquito larva and in the adult brain**

The expression of the different httN-QEGFP fusion proteins was visible under a fluorescence microscope. Interestingly httN-97QEGFP aggregates were observed in the larval brain, eye and ventral nerve chord according to their *3xP3* mediated expression **(Figure 5.19 A)**. In the dissected adult brain, the httN-97QEGFP aggregates were observed not only in the optic lobe but also in the sourrounding brain tissue (**Figure 5.19 B**). Overall the httN97-QEGFP expression pattern in the adult brain resembled that of httN-97QEGFP expressing *Drosophila melanogaster*

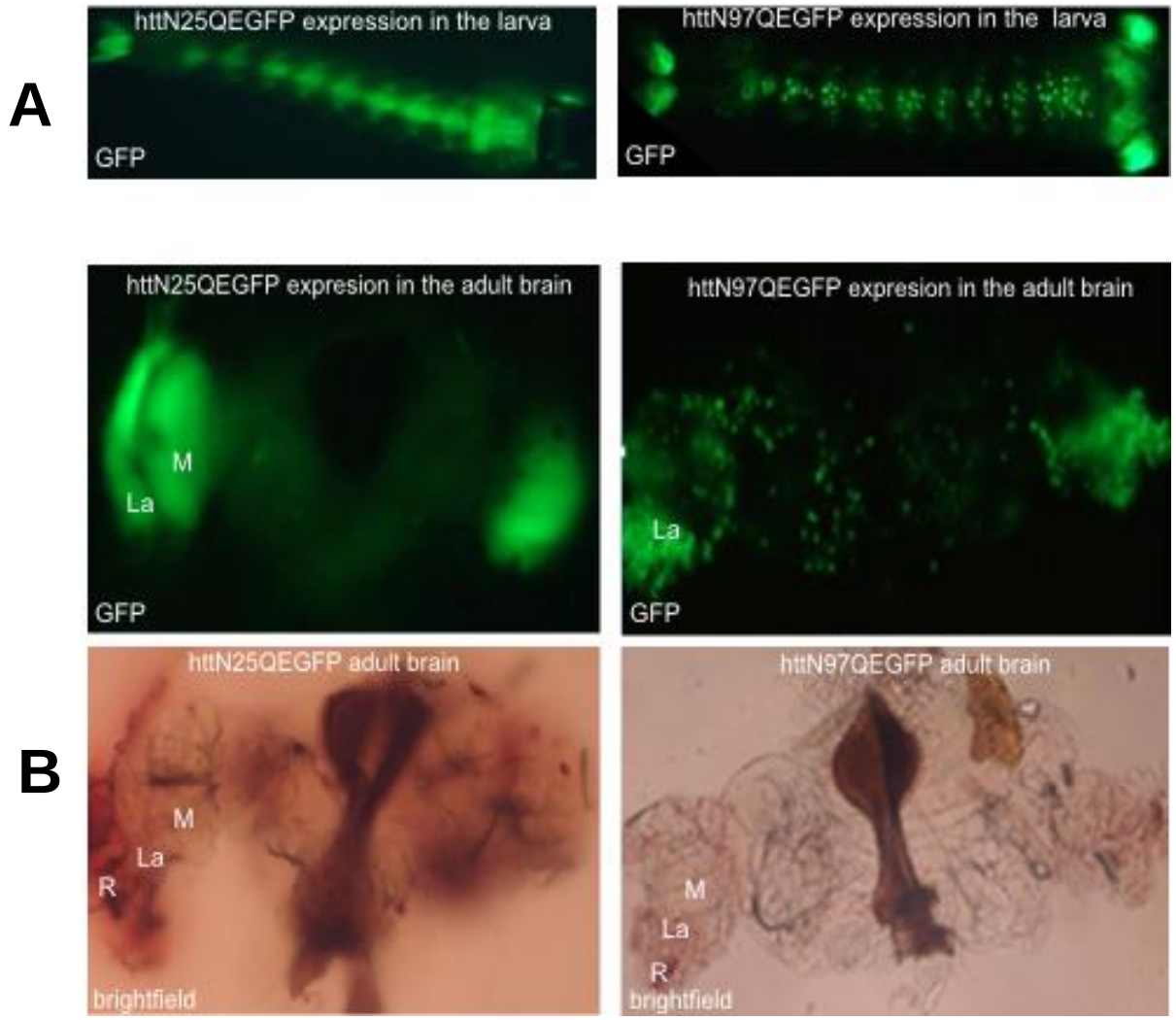


Fig. 5.19: HttN-97QEGFP forms aggregates in transgenic *An. gambiae* larva and adults. A *3xP3* mediated httN-25QEGFP and httN-97QEGFP expression in the larval ventral nerve chord, eye and anal papillae. **B** *3xP3* mediated httN-25QEGFP and httN-97QEGFP expression in dissected adult mosquito brains. Aggregate formation was seen only in larva and adult brain which expressed the httN97QEGFP protein. La- lamina, M- medulla, R- retina.

5.18. **HttN-97QEGFP expression reduces the larval but not the adult *An. gambiae* survival**

The 3xP3-mediated expression of httNQEGFP throughout life caused httN97QEGFP aggregate formation in all developmental stages of the mosquito. Since aggregate formation and neurodegeneration in Huntington's diseases is found to be age-dependent, it was therefore investigated whether and at what stage these aggregates have an effect on the mosquito survival. Firstly, in three different experiments the percentage of larvae that survived into adulthood was recorded for *An. gambiae* mosquitoes of the wildtype, the X1 parental line and the httN25QEGFP- and httN97QEGFP lines. Out of a total of ~400 L1-L2 larvae for each group on average 49% of the httN97QEGFP line emerged into adulthood, compared to 81, 83 and 68% for the controls (wildtype, X1 and httN25QEGFP respectively) (χ^2 test, $p< 0.05$) (**Figure 5.20 A**). This showed that *3xP3* mediated httN97QEGFP expression had a negative effect on the larval survival.

To examine any effects on adult life span, survival curves were obtained for both virgin females and virgin males (n=50) reared on 10% glucose (**Figure 5.20 B**). Virgins were chosen because mating can reduce the *An. gambiae* life span (Dao et al., 2010). Overall, no difference was found between the different groups (**Table 5.6**). Because aggregates are visible mainly in the optic lobe which could have interrupted the mosquitoes visual ability, the survival of adult mosquitoes under constant light (24h) was tested (**Figure 5.20 C, Table 5.6**). Males of the httN97QEGFP line lived significantly longer than males from the control group (log-rank test, $p\leq 0.05$) whereas no difference was observed between females (**Table 5.6**). Comparing the two light conditions httN97QEGFP females had a significantly longer median life span at 24h daylight than at 12h/12h day/night cycle (log-rank test, $p=0.02$). The survival of the control groups was not affected by the different light conditions. Therefore, because only changes in the mosquito survival were observed in httN97QEGFP mosquitoes it is possible that male and female mosquitoes have a changed light perception. However, from these two experiments it was concluded that in general the adult survival was not reduced by httN97QEGFP expression.

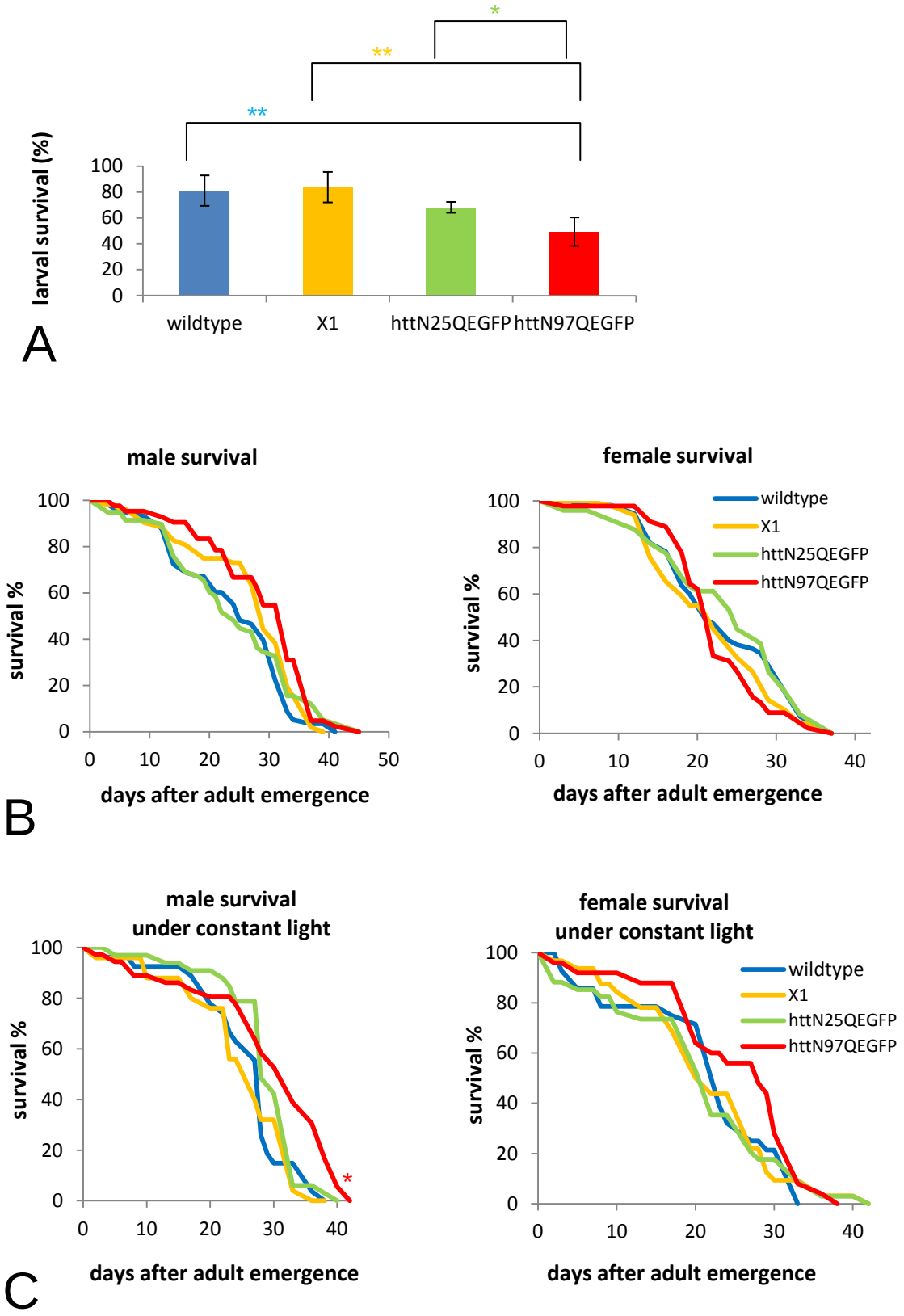


Fig. 5.20: HttN97QEGFP expression in *An. gambiae* reduces the larval and but not the adult life span. A Percentage of L1-L2 larvae which survived into adulthood (χ^2 test, * p = 0.03, **p=0.01). **B** Survival curves of male and female *An. gambiae* mosquitoes with a day and night cycle of 12h:12h **C** Survival curves of male and female *An. gambiae* mosquitoes under constant light (24h). Log-rank test of HttN97QEGFP male vs. controls, * $p \leq 0.05$.

Table 5.6: Survival analysis of adult male and female *An. gambiae* mosquitoes expressing httN97QEGFP.

			Experiment 1 (12h/12h day/night cycle)				Experiment 2 (24h day light)			
gender	line	polyQ	n	median (days)	maximum (days)	p-value (log-rank test)	n	median (days)	maximum (days)	p-value (log-rank test)
male	wildtype	0	58	25	41	0.002	27	28	38	0.004
	X1	0	52	29	39	0.085	25	27	36	0.003
	httN25Q-EGFP	25	58	24	45	0.089	33	28	38	0.054
	httN97Q-EGFP	97	42	33	45		36	33	42	
female	wildtype	0	55	21	37	0.362	28	23	38	0.287
	X1	0	49	22	37	0.563	32	22	36	0.163
	httN25Q-EGFP	25	49	25	37	0.143	34	22	40	0.195
	httN97Q-EGFP	97	45	22	37		25	28	42	

5.19. **Effect of httN-97QEGFP expression on mosquito behaviour**

Because httN97QEGFP females showed a change in survival depending on the light condition indicating a change in the optic lobe or the mosquito behaviour was investigated by performing a blood- feeding assay, recovery assay and chromatic oviposition assay.

The blood- feeding assay gave an indication of how the female is able to process host cues and whether it is able to feed on the host. A total of ~130-160 females of the wildtype, X1, httN25QEGFP and httN97QEGFP line were offered a mouse blood-meal for 10min in 6 independent experiments. The number of blood-fed females ranged on average between 62% (for httN97QEGFP) and 74% (for the wildtype). No significant difference between the different groups was observed (**Figure 5.21**).

Because httN97QEGFP flies showed reduced climbing behaviour, it was investigated whether the locomotory behaviour of mosquitoes expressing the same constructs could be affected in a similar way. Consistent with most animals it is generally observed that mosquitoes show decreased locomotory ability with age. However, mosquitoes do not climb as such. Therefore one approach was to assess the locomotory behaviour by recording the recovery time needed for mosquitoes to turn themself upright again after a CO_2 knockdown. 25QEGFP and 97QEGFP males and females were examined for the "turning response" at regular intervals for 24days. As expected all mosquitoes showed an age-dependent decline in their ability to recover from CO_2 treatment (**Figure 5.22**). However, no significant difference between the groups was observed.

In a third behavioural assay, it was investigated whether the httN97QEGFP aggregate formation in the adult optic lobe affected the function of the medulla projecting R7/R8 photoreceptors which enable colour vision. Mosquitoes distinguish between light intensity and wavelength (Clements, 1981). In general host-seeking female mosquitoes are more attracted to low-intensity colours such as blue, black and red than to high-intensity colors such as white and yellow (Browne and Bennett, 1981). Related to this, sensitivity of ovipositing *Aedes aegypti* mosquitoes to transmitted light of different wavelengths showed lowest acceptance to green to yellow-green light (532-572nm and highest acceptability to red (>616nm) (Snow et al., 1971).

In four experiments a total of 45- 49 females per line were blood-fed and four egg bowls coloured white, yellow, red and black were distributed randomly to each corner of the

mosquito cage. In all 4 experiments females of each line preferred to lay their eggs into red or black egg bowls rather than yellow or white egg bowls (χ^2-test, $p<0.0001$) (**Figure 5.23A**). Both, HttN25QEGFP and HttN97QEGFP females preferred black over red oviposition bowls (HttN25QEGFP χ^2-test, $p<0.01$, HttN97QEGFP χ^2-test, $p<0.0001$). From this experiment it was concluded that httN97QEGFP females are able to distinguish and chose between different colours.

It was then investigated whether mosquitoes could distinguish a certain egg bowl colour when it is surrounded by more than one different colour. The surrounding oviposition area had been observed to affect oviposition in *An. atroparvus*, whereas *An. gambiae* mosquitoes have been shown to lay their eggs preferentially in a petri dish with a black bottom independent of the surrounding surface (Kennedy, 1942, McCrae, 1984). In the following three oviposition assays the 4 different coloured egg bowls were placed next to each other and a total of ~ 27-34 females were allowed to choose between them after a blood-meal. All *An. gambiae* controls (httN25QEGFP, X1 and wildtype) laid 90-99% of their eggs in red or black oviposition bowl as observed in the previous experiments. However, in all three assays httN97QEGFP mosquitoes laid preferentially their eggs in the white egg bowl (**Figure 5.23B**). It is unclear how the surrounding light intensity and wavelength might have affected the egg laying behaviour of these females. This result has to be taken with caution because the average number of eggs laid by the httN97QEGFP group was relatively low compared to the other lines, which increases the chance that only a few females laid eggs and possibly into the white egg bowl (**Table 5.7**).

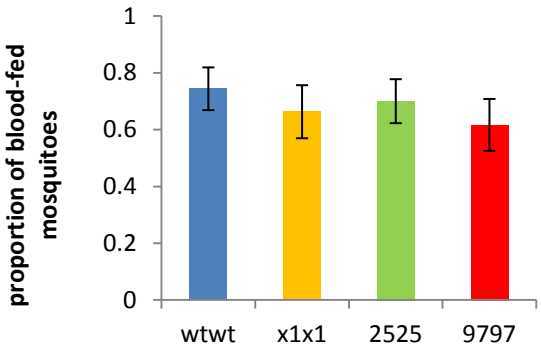


Fig. 5.21: Blood-feeding behaviour is unaffected in httN-97QEGFP mosquitoes. The proportion of blood-fed mosquitoes is represented as the proportion of females that fully engorged on a mouse within a 10minute period. Displayed is the average and standard error of the mean (χ^2 test $p > 0.05$).

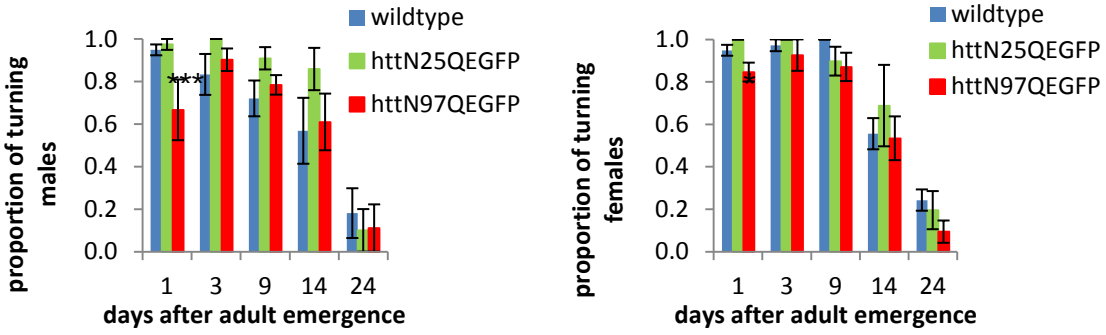


Fig. 5.22: The turning ability after CO_2 knockdown does not differ between httN-25QEGFP and httN-97QEGFP mosquitoes. Displayed is the average and standard error of the mean of 3 samples containing 13 male or female mosquitoes each. (χ^2 test, * $p<0.05$, *** $p<0.001$)

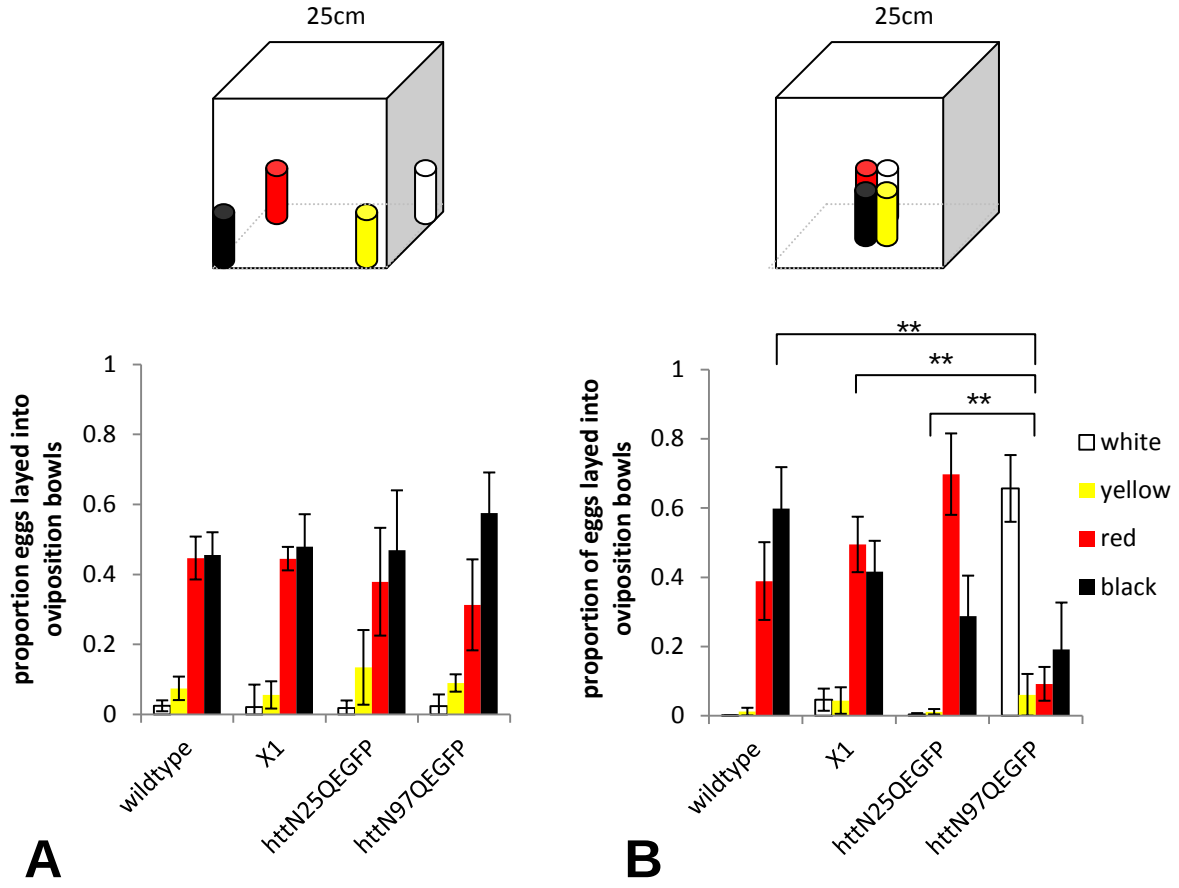


Fig. 5.23: HttN97QEGFP females change their oviposition behaviour depending on the area surrounding the egg bowl. A Displayed is the average proportion (±SEM) of eggs laid into different positioned coloured egg bowls by a total of ~46 females (4 Experiments; χ^2 test > 0.05 **B** Displayed is the average and SEM of the proportion of eggs laid from a total of ~30 females per group after placing the egg bowls next to each other (3 experiments; χ^2 test ** p< 0.001).

Table 5.7: Comparison of number of eggs laid into coloured egg bowls between httN97QEGFP and control lines

	experiment type A: egg bowls in different corners of a 25x25cm cage		experiment type B: egg bowls in the middle of a 25x25cm cage
mosquito line	Estimated mean number of eggs per female (4 repeats)	p-value (Mann-Whitney test)	Estimated mean number of eggs per female (3 repeats)
wildtype	36 (± 5)	0.06	52 (± 6)
X1	14 (± 26)	0.49	69 (± 26)
httN25QEGP	9 (± 34)	0.11	46 (± 6)
httN97QEGFP	8 (± 15)		6 (± 2)

5.20. **The fecundity of the httN-97QEGFP line is not reduced by expanded 97 polyglutamine expression**

Because groups of females expressing httN-97QEGFP were found to lay the fewest number of eggs in different coloured egg bowls, in the following oviposition assay it was investigated how many eggs are laid by single females. In three experiments 10 four-day old mosquitoes of the wildtype, X1, httN25QEGFP and the httN97QEGFP line were allowed to lay eggs into separate white filter paper covered oviposition bowls (**Table 5.8**). 30% of httN97QEGFP expressing females laid eggs compared to 40-50% for the controls. Females were believed to be sterile, if they laid eggs which did not hatch and were thus excluded from the analysis. HttN97QEGFP females laid more eggs than the X1 control. It was concluded that the oviposition and hatching rate is not reduced by expressing httN97QEGFP proteins in the mosquito's optic lobe and CNS.

Table 5.8: The reproduction potential of httN97QEGFP expressing females is not significantly reduced.

line	no. of fed females	no. of egg-laying females*	p-value (t-test of arcsine transformed proportion of females laying eggs)	mean no. of eggs (±SEM)	p-value (Mann-Whitney test comparing the number of eggs)	mean hatching rate in % (±SEM)	p-value (t-test of arcsine transformed hatching rate)
Wildtype (control)	30	14 (47%)	0.58	117 (± 8)	0.58	61 (± 4)	0.18
X1 (control)	30	12 (40%)		121 (±15)		53(± 4)	
httN25QEGFP (control)	30	15 (50%)	0.87	126 (±11)	0.87	56 (±5)	0.68
httN97QEGFP	30	9 (30%)	0.49	160 (±7)	0.01	47(± 5)	0.36

*Females with at least one hatching egg (2 Females of the X1 line and httN25QEGFP line were excluded from the analysis).

5.21. **Development of vectors containing (CAG/CAA)n GFP fusion proteins driven by the *Dmelav* promoter**

Despite characteristic aggregate formation of httN97QEGFP proteins in the adult optic lobe, no visible neurodegeneration of the eye was observed in the httN97QEGFP fly or mosquito lines. One reason for this could be that the 3xP3 promoter expressed the httN97QEGFP not in neurons which are especially prone to neurodegeneration in HD disease. Previously, Zhang et al. showed that under the panneuronal Dm*elav* promoter expression of httN72QEGFP and httN103QEGFP caused an age and polyQ- dependent formation of aggregates loss of eye pigmentation at day 30 indicating degeneration of underlying eye tissues at this stage. Because, *elav* has been a widely used for the expression of polyQ proteins, one strategy was to replace the promoter *3xP3* of the two httNQEGP constructs with this fly *elav* promoter. This fly promoter was assumed to be suitable due to high homology of the *D. melanogaster* and *An. gambiae* ELAV protein (**Appendix Figure 10.6**). Further, immunostaining of the *An. gambiae* brain with the DmELAV-antibody showed staining of the nuclei of neuronal cells counterstained with DAPI and indicates a similar function of ELAV in flies and mosquitoes (**Figure 5.24 A-G**).

For transgenesis 1256 and 2312 embryos were injected with the elav-httN25QEGFP or elav-httN97QEGFPvector and the vasa integrase helper plasmid. In total, only 9 and 10 elav-httN25QEGFP and elav-httN97QEGFP injected adults survived, but none of the 2235 and 4152 screened progeny showed transgenic elav-httNQEGFP expression.

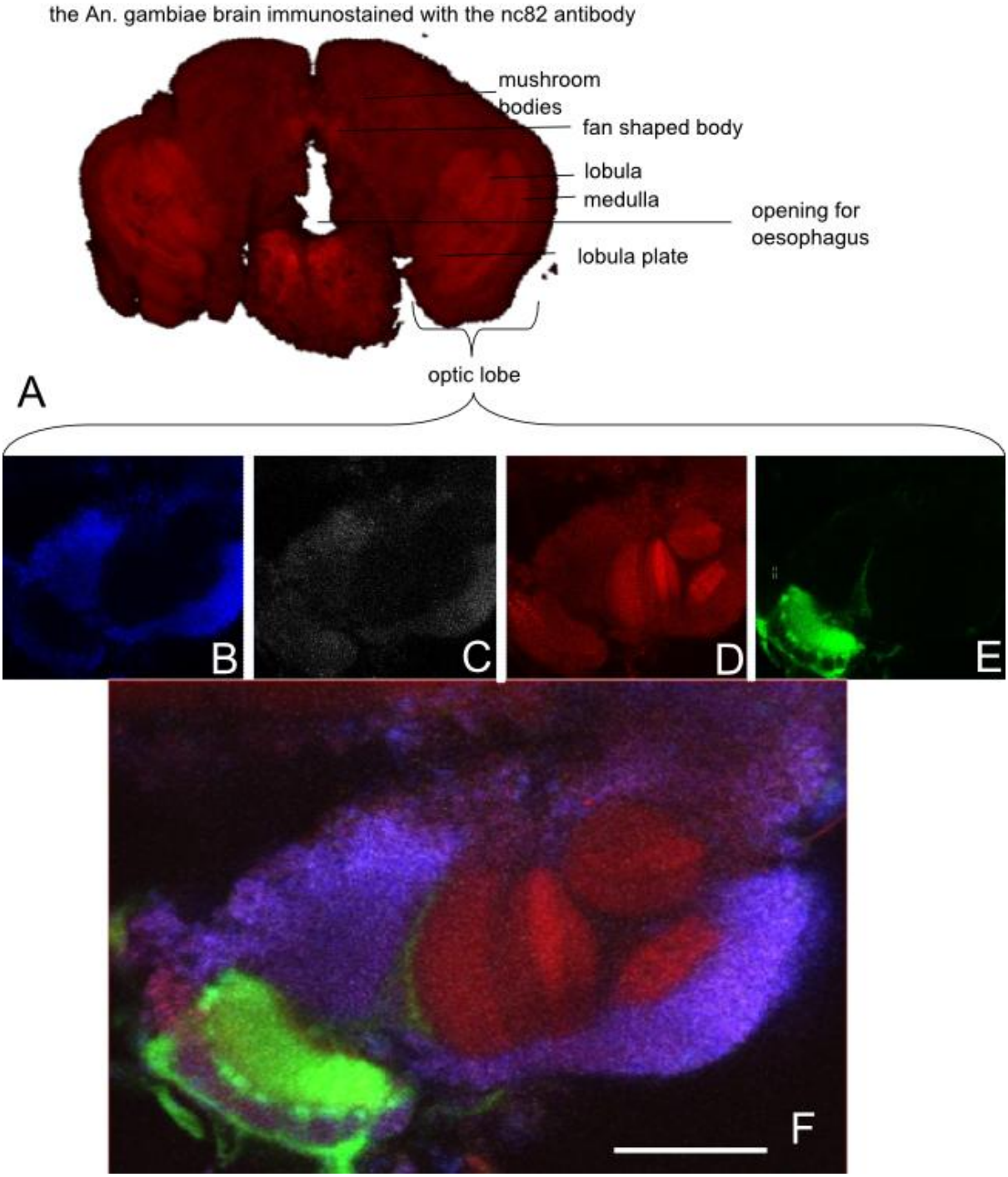


Fig. 5.24: Immunostaining of the httex1p52Q *An. gambiae* brain. A Overview of the *An. gambiae* brain immunostained for the neuropil marker nc82. **B** DAPI staining nuclei of the optic lobe **C** DmELAVstaining specifically neuronal nuclei of the optic lobe **D** Nc82 antibody staining the neuropil of the optic lobe **E** Transegnic 3xP3 driven EGFP expression staining the photoreceptor cells of the optic lobe. **F** Picture overlay of B-E. Green: EGFP, red: nc82, purple: Dapi, nc82 and Anti-ELAV. Scale bar 100µm

5.22. **Identification of the transcriptional start sites of the neuron-specific *ddc* gene**

So far the expression of neuronal effector genes like pathogenic huntingtin fragments is restricted to only one established neuron specific promoter (3xP3) in *An. gambiae*. Another alternative promoter candidate is *ddc*. In a *D. melanogaster* Parkinson model the endogenous promoter has been previously used to model this neurological disease. Further this promoter contains regulatory regions that express the *ddc* gene either in dopaminergic or serotinergic neurons (Johnson et al., 1989). Both of these neurons might be affected differently by Huntingtin expression. *Anopheles gambiae* shares a high homology with *D. melanogaster* DDC which suggests a conserved function (75%, **Appendix Figure 10.7**).

In order to establish the amount of upstream sequence necessary to ensure the correct recapitulation of expression a 5' RACE was performed to determine the transcriptional start site of *An. gambiae ddc*. The RACE PCR and the sequencing of the PCR products revealed two transcripts with two different transcriptional start sites. The start site of the larger fragment was 1533bp upstream of the ATG of the proposed ddcRA transcript, whereas the smaller start site was 139bp upstream of the ATG start of the ddcRB exon1 (**Figure 5.25 B,C**). The proposed exon structure and start site of the two transcripts is shown in **Figure 5.25 D**. In *Drosophila*, the different *ddc* transcripts are expressed in the hypoderm and CNS, possibly these two transcripts refer to the expression in a different tissue too (Chen et al., 1993). In the future the tissue-specfic expression will have to be characterised in the different mosquito body tissues in order to determine the correct 3' promoter end for the neuron specific expression.

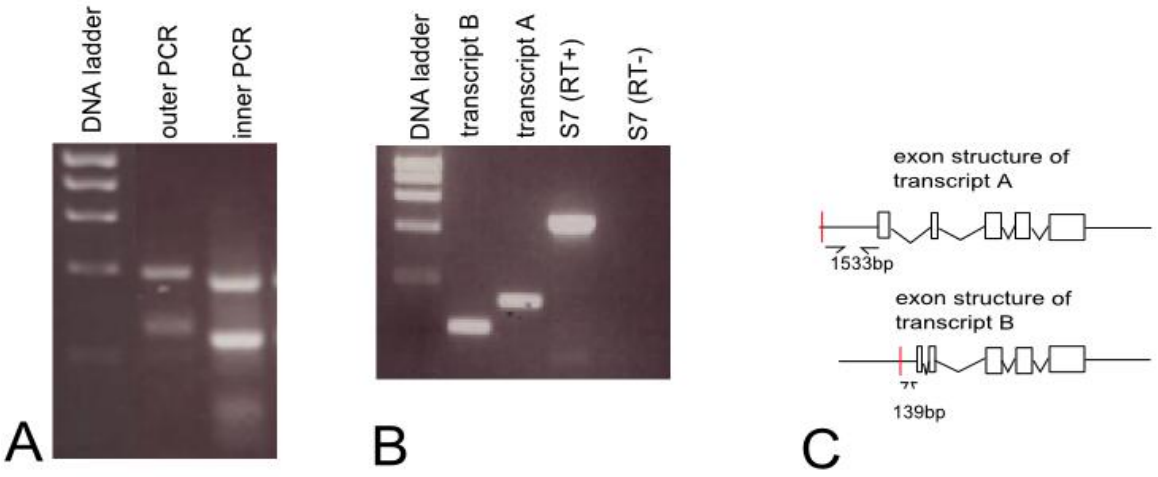


Fig. 5.25: Identification of the transcriptional start sites of DDC. A Principle of the 5' RACE **B** Outer and inner PCR using 5' RACE adapter specific and ddc specific primers **C** PCR of mosquito cDNA amplifying the two ddc fragments. **D** Proposed exon structure and transcriptional start sites of the two *ddc* transcripts (shown not to scale).

6. Chapter: Inducing a polyglutamine disease in *Anopheles gambiae* mosquitoes - Discussion

Several polyglutamine diseases like HD have been induced in model organisms like *D. melanogaster* which led to neurodegeneration and a shortened life span under the regulation of different neuron and eye-specific promoters such as *rhodopsin, gmr* or *elav* (Sang et al., 2005, Godin et al., 2010, Wolfgang et al., 2005).

The *3xP3* promoter has been widely applied in *Anopheles gambiae* for marker expression in the larval and adult eye and was chosen for expression of htt-exon1 fragments that featured polyglutamines stretches of putative pathogenic or non-pathogenic lengths. Unfortunately, several generations after the transgenic httex1p20Q, httex1p52Q and httex1p69Q *An. gambiae* lines were established they lost their pathogenic polyQ length leaving only 21PolyQ repeats. This instability of CAG repeats of htt has been reported in prokaryotes and eukaryotes like humans and *Drosophila* (Gusella and MacDonald, 2000, Marsh et al., 2000, Scherzinger et al., 1999). Indeed, retrospectively, the loss of repeats was also observed in bacterial culture of the plasmids containing the large polyQ stretch. However, it was not known how fast this would occur in a mosquito population. The mechanism of CAG repeat change is thought to be a DNA replication slippage which is initiated by hairpin folding of the CAG repeat template (Mitas, 1997). In humans, polyQ length between 5 and 24 are normal. Intermediate-HD alleles having 25±34 repeats are genetically unstable and can expand to hundreds of repeats, which exhibit a high degree of genetic instability. The tight association between repeat lengths with mutation is called “dynamic mutation”. It describes the likelihood for the product of an expansion mutation to undergo subsequent mutation relative to the precursor allele. The increased instability of longer lengths is thought to be due to an increased ability to form mutagenic DNA structures such as DNA hairpins.

Interestingly, all three *An. gambiae* lines contained after several generations either 24 (httex1p20Q) or 21 (httex1p52Q and httex1p69Q) polyglutamine stretches. In similar *Drosophila* studies Marsh observed a reduction from 108PolyQs to 22 PolyQs (Marsh et al., 2000). In addition to this, a blast search on natural *An. gambiae* polyglutamine proteins with more than 20 PolyQ repeats revealed that they contained no more than 22 continuous CAG stretches. Therefore, a CAG repeat length of ~ 22 continuous stretches might reduce the

ability to form these hairpins and therefore prevent slippage of repeats during DNA replication.

Different antibodies were unable to detect the huntingtin exon1 protein in transgenic lines by western blotting, despite several modifications. Similarly to this in the literature problems have been reported to detect polyglutamines with antibodies against mutated htt exon 1 and long polglutamine stretches (Sugaya et al., 2007; Gunawardena et al., 2003). One reason for this is the tendency of the mutated htt proteins to form SDS- resistant aggregate, whereas their soluble forms are located in the nucleus (Sugaya et al., 2007, Lunkes et al., 2002). Gunawardena detected extended polyglutamines only if they were associated with a nuclear export sequence (NES) (Gunawardena et al., 2003).

In all Western blots not only a smear (found in previous studies) but also distinct bands were observed in the control and in the httex1pQ lines. A Blast search revealed that *Anopheles gambiae* contains at least three natural polyQ containing proteins with more than 38 continuous PolyQ stretches (max 132Q) which could have potentially cross hybridised with the htt antibodies (**Appendix Table 10.8**). In *D. melanogaster* other polyQ containing proteins were identified but did not have such a high number of repeats (for example echinus has 34PolyQs). These *An. gambiae* polyQ proteins: AGAP006758, AGAP005135, AGAP004734 have been potentially a DNA binding function and are expressed ubiquitously (NCBI database; Marinotti et al., 2005). However, although transcripts of these genes have been found, their overall sequence especially the long polyQ stretch has not been supported by ESTs (Vectorbase database). Further, orthologue genes in other organisms shared only low similarities and no PolyQ stretch with these proteins (Vector base, NCBI database).

In terms of polyglutamine toxicity, the fact that *An. gambiae* might have polyQ proteins with >35 PolyQ repeats raised three hypotheses: a) *An. gambiae* has a mechanism that prevents neurodegeneration caused by polyQ containing proteins; b) polyQs are not the only important component to induce pathogenesis; c) because all polyQ stretches of these endogenous non-pathogenic proteins consist of not more than 22 continuous CAG repeats that are interrupted by CAA repeats also encoding glutamine, possibly the RNA rather the protein is the toxic agent causing this disease.

In order to address the first point a survival experiment was performed which showed that female httex1p52Q mosquitoes expressing polyglutamines of a pathogenic length of 52 repeats had a reduced life span compared to the attP line, httex1p20 and httex1p69 which had

lost its repeats at the time of this experiment. This result was promising but unfortunately could not be repeated due to a loss of 31 polyQ repeats of the httex1p52Q line at later generations.

The second point is more likely though for the following reasons: polyglutamine toxicity is context (tissue-) dependent and proline-rich regions of *htt* and exogenous Flag tags are able to modify the polyQ toxicity (Duennwald et al., 2006). While epitope tags reduced the toxicity of expressed polyglutamines, proline stretches had the ability to antagonise the toxicity but also promoted toxic conformations in 103Qhtt proteins under certain circumstances. This is interesting because none of the endogenous *An. gambiae* polyglutamine containing proteins contained these proline stretches, suggesting that they might be important for pathogenesis.

The third hypothesis which assumes that the expanded RNA rather than the protein might be the pathogenic agent is controversial. In a polyglutamine disease fly model Li et al. investigated the role of the expanded ataxin-3 RNA (Spinocerebellar ataxia type 3) by altering the CAG repeat sequence to an interrupted CAG/CAA repeat within the polyQ-encoding region and found that this dramatically mitigated toxicity (Li et al., 2008). Because this confirmation only changed the DNA/RNA but not the protein sequence (both CAG and CAA encode glutamine), it was assumed that pathogenesis expanded CAG repeats is caused mainly by mutated RNA. The toxicity could be for example associated with their localisation in the nucleus which suggests that the mRNA might adopt ds-like RNA conformations. In support of this mutated RNA from several polyglutamine disease causing genes including huntingtin has been found to colocalise with proteins such as muscleblind (MBLN-1) in nuclear inclusions in several polyglutamine diseases (Mankodi et al., 2001, Li et al., 2008, de Mezer et al., 2011). However, despite a potential role of RNA in HD disease, fly HD models expressing N-terminal expanded htt- CAG/$CAA_{>35}$ proteins observed increased lethality and disruption in the eye pigment formation which supports the hypothesis that the protein is pathogenic (Weiss et al., 2011, Zhang et al., 2010).

Because of the observed problems of DNA instability and protein detection a new strategy was to express more stable htt-N fragments with alternating $(CAGCAA)_n$ in *An. gambiae*. In addition to this these httN-Q constructs were fused to EGFP to allow an easy detection under a fluorescent microscope. However, bearing in mind the possibility that RNA rather than DNA might be toxic and the novelty of using the *3xP3* promoter to express htt, firstly the expression of these constructs was validated in a transgenic fly model. It was shown that

these proteins form aggregates in the fly brain and reduce their adult life span and climbing ability. Interestingly, when the same proteins were introduced into mosquitoes, similar aggregates in the brain were found throughout the larval CNS and in the mosquito brain, but an increased mortality was observed for larvae but not for adult mosquitoes. The fact that no effect on adult mosquito survival was seen despite characteristic aggregate formation supports the hypothesis that aggregate formation might not be the cause for expanded huntingtin pathogenesis (Romero et al., 2008, Slow et al., 2005). One reason of why there was a lethal effect in the larvae but not in the adult mosquito could be due to the expression pattern of the *3xP3* promoter. This promoter was shown to express fluorescent marker genes in the larval eye, ventral nerve chord and anal papillae. Compared to this in adults fluorescence was mainly observed in the eye and sporadically in the nerve chord. It also possible that compared to the control strains, the increased larval mortality led to decreased competition and higher food availability for the remaining larvae, which promoted the survival during adulthood. Another reason of why adult mosquitoes were not affected but adult flies could be that these mosquitoes died of another cause of mortality which decreased their life span and masked the putative toxicity of expanded htt. It was for example observed that all female mosquito lines lived shorter than males, whereas the opposite was the case for flies. One reason for the reduced female mosquito life span could be that females were lacking a protein source in their food. There are studies which found that female Anopheline mosquitoes lived longest on a blood and sugar meal, than on sugar or blood alone (Okech et al., 2003). Therefore, the blood meal might not be only a source for egg production but also necessary for the mosquito survival as energy source (Takken et al., 1998).

This assumption does not explain why adult httex1p52Q females showed a reduced life span. In contrast to httN97QEGFP females these mosquitoes expressed continuous CAG stretches and no GFP marker gene. It is therefore possible that httN97QEGFP mosquitoes lacked RNA mediated toxicity due to alternating CAG/CAA repeats or that the GFP tag reduced the toxicity of the polyQ repeats. Various protein tags have been shown to mitigate toxicity in other HD *Drosophila* models (Marsh et al., 2000).

Despite the absence of an effect on adult survival, it was investigated whether the expression of httN97QEGFP by the eye and neuron-specific promoter *3xP3* would affect the vision and behaviour of these mosquitoes. While no change in blood feeding behaviour or recovery after CO_2 exposure was found, the oviposition behaviour was changed when females were allowed to choose between a set of coloured egg bowls which were positioned next to each other. In

general, mosquitoes prefer laying their eggs in low intensity coloured oviposition bowls that are black or red over ones that are covered with high intensity colours such as yellow or white (Kennedy, 1942). It was expected that mosquitoes with an abolished vision would be less able to distinguish between these colours resulting in an equal number of eggs laid in each egg bowl. Instead, httN97QEGFP females preferred white over black, red and yellow egg bowls, whereas the control lines least preference was as expected white or yellow. This was unusual especially because httN97QEGFP females preferred black and red like the control lines when the egg bowls where placed separately in different corners of the cage. Therefore a change in the surrounding area of the egg bowl was believed to be responsible for this change. It is possible that white which has the highest intensity and reflection of light was most distinguishable from the other colours for these mosquitoes. Nevertheless, caution must be exercised in relating positive or negative responses to starkly black and white targets to behaviour of wild mosquitoes (Clements, 1999).

It remains to be investigated whether there also other behavioural defects which could possibly influence the mosquito transmission potential.

All together it can be concluded that expression of expanded httN97QEGFP can be pathogenic in Anopheline mosquitoes, but its toxicity might be reduced by the alternating CAG/CAA repeats, the GFP tag and an unsuitable promoter. Other promoters like *elav* or *ddc* were partially investigated in this thesis and could potentially express httN97QEGFP in all neurons or susceptible neurons and might cause a more severe phenotype that leads to a decreased adult life span of Anopheline mosquitoes. Further, expression by the non-neuronal *dpp* promoter in epithelia of imaginal discs led to missing legs, abnormal head capsule evagination and development of the mesothorax (notum) showed that toxicity of expanded polyglutamine fragments might not be limited to neurons (Marsh et al., 2000). Therefore, possibly nonneuronal blood meal- responsive promoters like vitellogenin that limit the expression to adult mosquitoes could be future promoter candidates.

7. **Chapter: Conclusions**

In the past decade there have been reductions in reported malaria cases of more than 50% in 43 of the 99 countries with ongoing transmission attributed mainly to effective malaria control interventions like ITNs and ITSs as well as artemisinin-based combination therapies (WHO, 2011). The next few years will be critical in the fight against malaria, because insecticide treated bed nets will need to be replaced and parasite resistance to antimalarial medicines threatens the achieved gains (WHO, 2011). Furthermore, in 2010 resistance to pyrethroids, the only class of insecticides used on insecticide-treated mosquito nets, has been reported in 27 countries of sub-Saharan Africa (WHO, 2011). In order to sustain improvements of malaria control, new effective insecticides, vaccines and durable ITNs (current life time is 3 years) have to be developed and deployed, oral monotherapies have to be withdrawn. One problem of existing insecticides is that they act immediately which makes them effective vector control tools but also puts a high selection pressure on young reproductive females towards development of resistance. Therefore, in order to achieve sustainable vector control, new class of insecticides could be developed that kills mosquitoes before the parasite enters its infectious stage but late enough to reduce the effect on fecundity which could slow down the evolution of resistance. There are potential late-life acting biopesticides like entomopathogenic fungi, microsporidia and densoviruses which replicate within the host throughout the infectious period and increase in their density-dependent toxicity with host age, thus affecting rather old mosquitoes (Lorenz and Koella, 2011, Ren and Rasgon, 2010, Blanford et al., 2011). However, the parasite growth rate and thus pathogenity of these biopesticides depends highly on the parasite infectious dose and host condition (food availability, temperature or seasonal changes) (Timms et al., 2001, Lorenz and Koella, 2011). Both factors are subject to little (for example number of times a female mosquito rests on a biopesticide impregnated bed net) or no control (for example climate) under non-laboratorial conditions. Another concern is the possibility of infection of non-target species especially in the aquatic habitat, which is typical for larvae of many insect orders (McCafferty, 1981).

One alternative approach to increase late-life mortality specifically in Anopheline mosquitoes which does not involve density-dependent biopesticides relies on genetic technologies. In 2010, Corby-Harris and colleagues generated genetically transformed *Anopheles stephensi*

mosquitoes by overexpression of an endogenous Akt kinase which led to reduced mosquito infection with *P. falciparum* and a shortened adult mosquito life span (Corby-Harris, 2010).

In this study two new transgene-based approaches were investigated. One strategy was to induce a lethal amino acid disease in response to an adult female blood meal by knocking down enzymes involved in the metabolisation of ingested phenylalanine and tyrosine. As discussed, an accumulation of phenylalanine caused by a knockdown of the *An. gambiae* phenylalanine hydroxylase did not lead to an increased mortality after blood meal. This was potentially due to the lack of sufficient pathogenic phenyllactate production, which has been observed to be toxic in PKU patients. In the future, enzymes involved in metabolising other amino acids which are more abundant in the ingested blood could be targeted. However, one problem with this strategy is that because females rely on a blood meal in order to lay eggs killing females after the first blood meal, as shown by the knockdown of PPO9 would pose a high selection pressure for the development of resistance and no improvement compared to conventional insecticides. Therefore, it is essential that the accumulation of toxic metabolites increases with the number of blood meals, whereby the first blood meals should leave the mosquitoes unaffected. Further, a stable transgenic knockdown under a blood meal dependent promoter like vitellogenin could ensure that amino acid metabolism during larval stage is unaffected. However, in this thesis a knockdown of PAH did not lead to increased adult mortality even after repeated blood meals, which showed that this strategy is potentially difficult to implement.

Another strategy was to induce a blood-meal independent neurodegenerative disease by transgenic expression of human huntingtin fragments with expanded polyglutamine stretches that would accumulate in the mosquito brain and lead to its death. While in flies adult survival and climbing ability was reduced no life span decreasing effect was observed in adult mosquitoes. However, *3xP3* promoter-mediated expression increased mosquito larval mortality. New promoters which express polyglutamine constructs stage- and tissue specific and kill solely adult mosquitoes will need to be identified. In fact, all strategies which involve the genetic transformation of mosquitoes to make them refractory to malaria transmission will depend on suitable stage, tissue and potentially sex-specific expression of the anti-malarial transgenes. In contrast to other potential late-life acting transgenic approaches that rely on overexpression or knockout of genes, the polyglutamine disease approach has the advantage that the construct is highly modifiable by varying polyglutamine length which correlates with onset and severity of the disease. Therefore, having a construct

like polyglutamines which is easily adjustable will be of advantage when assessing the effectiveness of this approach outside the lab environment.

Most likely all biopesticide or transgene based vector control approaches will lead to some fitness costs for affected mosquitoes which hinders the spread of the desired trait within a wild mosquito population. However, some strategies will lead to stronger fitness costs than others. SIT, RIDL or embryonic lethality strategies which are based on releasing sterile males or males which carry lethal factors that kill female offspring aim to suppress vector populations and pose a high fitness cost by reducing the number of offspring between 50-100%. In contrast killing mosquitoes at the fourth gonotrophic cycle using late-life acting biopesticides or transgenes would eliminate only ~ 22% of the progeny, compared to 85% kill on first contact insecticides and assuming a gonotrophic cycle every 2-3 days still inhibit malaria transmission (Read et al., 2009, Gillies and Wilkes, 1965). In addition the expression of a resistance gene which would make the mosquito refractory to the parasite will result in fitness costs (Moreira et al., 2004). Using transgenic technologies, one way of overcoming fitness costs and to minimse the risk of development of resistance could be by using a strong gene drive system which would spread and fixate for example the late-life acting transgene into mosquito populations. However, even then a decrease in malaria prevalence could only be expected if refractoriness is nearly 100% effective (Koella and Zaghloul, 2008, Read et al., 2009).

All transgenic technologies face the challenge of public acceptance. Inserted genes which express toxic proteins like huntingtin in mosquitoes could be also pathogenic in humans. Transgenic constructs may be remobilised, cross species barriers and inserted into the human genome. The risk of introduction of mutated toxic htt into the human host was minimised by choosing the *3xP3* promoter that expresses the httNQEGFP proteins in tissues like the mosquito eye and neurons which are not in contact with human blood and by using the stable attB/attP germline transformation system to generate transgenic mosquitoes.

There are further general concerns regarding the public acceptance of late- life acting vector control approaches which allow young non-transmitting females to bite people and reproduce, instead of killing them immediately (Dolgin, 2009). Additionally, there are risks associated with the evolutionary adaptation of mosquitoes to late-life acting agents. Mosquitoes with a shorter adult life span could be able to reduce transmission in the short term but may select for a more rapid sporogenic cycle of *Plasmodium* parasites. This could

have negative effects for the mosquito vector. A more rapid development of the sporogenic cycle could be possibly associated with a change in numbers of mature sporozoites which have been shown to affect mosquito biting frequency and mortality (Koella and Packer, 1996, Koella et al., 1998, Anderson et al., 2000). Changes in the mosquito life traits such as the human biting rate and life span could in turn affect the mosquito fecundity, which might put additional selection pressure on the mosquito to develop resistance against the life-shortening transgenic construct or insecticide (Rossignol et al., 1986, Koella et al., 2002).

Despite this, it is surprising that the parasite has such a long EIP of 10-14 days regarding the high vector mortality caused naturally and by insecticides. High extrinsic mortality rates of the host, independent of the parasite, are assumed to cause a high selection pressure towards more virulent parasites (Ebert and Weisser, 1997, Choo et al., 2003, Williams and Day, 2001). One reason of why this has not readily observed in natural vector-borne pathogen systems could be that many sporozites are needed in the salivary glands for transmission to occur (Paul et al., 2003). As such, it is likely that selective pressures imposed by the vector and vertebrate host may act in opposing directions. Experiments using rodent malaria have demonstrated that higher parasite virulence can be selected relatively quickly when parasites are sequentially passaged through just a mammalian host (Mackinnon et al., 2005). However, selection was found to be much less effective when parasites were alternated between mammalian and insect hosts (Cook et al., 2008).

Therefore, despite the mentioned challenges associated with this approach this strategy has the merit of being “evolutionary sustainable” and could be therefore a good addition to a variety of current and future vector control strategies which aim to suppress or to replace vector populations. It is now widely accepted that successful malaria control will not rely on one technique but involve a combination of different approaches (Ramirez et al., 2009).

8. **Future work**

The two approaches introduced here provide excellent models to investigate the effects of potential life span reduction as well as behavioural changes and reduced melanisation on malaria transmission.

Different amino acid disease causing enzymes could be knocked down and their effect on mosquito life span after several blood meals could be tested. Further, it needs to be tested whether the reduced melanisation of *P. berghei* ookinetes in dsPAH injected mosquitoes could be recapitulated using the human malaria parasite *P. falciparum*. In addition to this, feeding of octopamine could possibly reverse the oviposition arrest observed in dsPAH injected females and could identify an important regulator for octopamine synthesis in *Anopheles gambiae*. This could be useful for potential new strategies to interfere with this neurotransmitter that is known to be involved in learning and memory in *D. melanogaster* (Unoki et al., 2005, Schwaerzel et al., 2003). Further, in a transgenic model it could be tested whether overexpression of CLIPB9 a negative regulator of the PPO cascade could lead to predicted decrease in survival and increase in melanisation.

3xP3 mediated expression of htt fragments with expanded polyglutamines was pathogenic in a transgenic HD mosquito model and led to reduced larval survival. It is possible that increased larval mortality led to decreased competition and increased food availability which potentially provided an advantage for the remaining survivors throughout adulthood; therefore the adult survival experiments need to be repeated with larvae raised in single wells under controlled food availability and optimal humidity conditions (ideally ~80%, http://www.mr4.org). Oviposition behaviour differed in httN97Q expressing mosquitoes from the control strains depending on the surrounding colour of the oviposition bowl. In order to investigate whether these mosquitoes have a modified contrast vision rather than specific colour perception oviposition into black or white oviposition trays with black or white background could be investigated (Huang et al., 2007). It will be also of importance to investigate whether polyglutamine expressing females have a changed feeding behaviour due to a potentially different light perception.

Contrast vision might not only be important for oviposition behaviour of females but also influence the male swarming behaviour which has been shown to be important for mating in several experimnets. In a field study in São Tomé, Charlwood and colleagues showed that

male *Anopheles gambiae* swarming could be induced to follow an articial 1.0 x1.5m black marker (Charlwood et al., 2002). Swarms failed to form in the absence of an edge. Nevertheless the role of swarming needs to be explored in more detail, as other studies suggested that no swarming is required for mating success (Lounibos et al., 1998).

In order to to induce late-life mortality in *An. gambiae* new suitable polyglutamine driving promoters need to be generated. However, the generation of transgenic mosquitoes remains time consuming and limits the number of potentially generated new promoters. Recently, the Gal4/UAS system has been improved in *Anopheles gambiae* cell line and could be a very useful tool for the transgenic mosquito technologies in future (Lynd and Lycett, 2011). This system which has been successfully applied in *D. melanogaster* models, uses a driver line containing the transcativator protein GAL4 under control of a specific promoter and a responder line that contains GAL4 binding sites or upstream activation sequences (UAS) located 5' of the target gene (Fischer et al., 1988; Brand & Perrimon, 1993). Crossing of these 2 lines results in target gene expression under the specific promoter in the progeny. If applied to *An. gambiae* this system would allow studying the phenotypic effects of expression of Htt constructs or other anti-Plasmodium gene under control of different promoters without creating new transgenic lines for each promoter gene combination. Further, even in case a very strong promoter leads to immense fitness costs caused by expression of these genes in mosquitoes, the transgenic strains can be maintained without these fitness effects, because the responder line would not be able to express the transgene without the Gal4 driving specific promoter from the driver line. Therefore, this system and other technologies like targeted gene knockout system will open up new population suppressing or replacing strategies using transgenic technologies. Potentially future target genes will be involved in mosquito behaviour and life span control.

9. **References**

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10. Appendix

Appendix Table 10.1: Genes/ proteins that cause a shortened life span in *S. cerevisiae, C. elegans, M. musculus* and *H. sapiens*

cellular mechanism	**gene/ protein name**	**organism**	**phenotype**	**form of mutation**	**function**	**References**
cellular maintenance	*ATM* (Ataxia-telangiectasia)	*H. sapiens*	shortens life span to max. 52 years, cerebrellar ataxia from early childhood to progressive neurologic degeneration, irradiation sensitivity, increased risk of tumors	loss of function, recessive	checkpoint kinase in response to ionizing radiation, phosphorylation of multiple proteins incl. p53	(Banin et al., 1998, Canman et al., 1998)
	bec-1 (Beclin1)	*C. elegans*	shortens life span by 14%	RNAi	involved in autophagy	(Melendez et al., 2003)
	BLM (Bloom syndrome)	*H. sapiens*	shortened life span, death of cancer (leukaemia and lymphoma), growth deficiency, sun-sensitivity	loss of function	DNA helicase of the REC family,	(Ellis et al., 1995)
	BubR1	*M. musculus*	shortened life span by 60%, dwarfism, cataracts, impaired wound healing, Mosaic variegated aneuploidy (MVA)	loss of function	spindle assembly checkpoint protein	(Baker et al., 2004)

	CDC7 (cell division cycle)	*S. cerevisiae*	shortened life span,	loss of function, recessive	initiation of DNA replication, silencing	(Egilmez and Jazwinski, 1989)
	CKN-1 (Cockayne)	*H. sapiens*	Cockayne syndrome (CSA), shortened life span, retinal degradation, mental retardation	loss of function, recessive	WD40 repeat protein, corrects UV sensitivity in CSA cells	(Henning et al., 1995)
	CTF4	*S. cerevisiae*	shortens life span by 75%	loss of function	DNA polymerase alpha binding protein involved in regulating replication initiation	(Hoopes et al., 2002)
	Dmp53	*D. melanogaster*	slightly shortens life span at later ages, sensitive to irradiation	loss of function, recessive	Tumour suppressor/DNA-binding transcription factor important for apoptosis in mammals	(Lee et al., 2003a)
	DNA2	*S. cerevisiae*	decreased life span by up to 85%	loss of function	DNA replication helicase	(Hoopes et al., 2002)
	drd (Drop-dead)	*D. melanogaster*	die early in adult life (within 2 weeks of eclosion), gross neuropatholgical lesions in brain, may cause accelerated aging, x-chromosomal recessive, abnormal behaviour	loss of function, recessive	provide glial function (prevents neurodegeneration)	(Buchanan and Benzer, 1993, Blumenthal, 2008)

	HDF1 and *HDF2*	*S. cerevisiae*	shortened life span	loss of function	Non-homologous end-joining, telomere maintenance	(Kaeberlein et al., 1999, Milne et al., 1996, Laroche et al., 1998)
	HPR5(hyper-gene conversion)	*S. cerevisiae*	shortened life span by up to 60%	loss of function	DNA helicase	(McVey et al., 2001)
	hsp70 (heat shock protein)	*D. melanogaster*	enhances polyglutamine toxicity	loss of function	recovery of normal RNA processing and protein synthesis, protein protection by binding extended hydrophobic amino acid sequences that are normally sequestered in the protein core, suppressing ataxin-3 toxicity	(Warrick et al., 1999, Chan et al., 2000)
	human tau (wildtype)	*D. melanogaster*	shortened life span, adult onset, progressive neurodegeneration, accumulation of abnormal tau, Neurofibrillar protein aggregates, inhibits kinesin-dependent trafficking of vesicles, mitochondria, and Endoplasmic Reticulum neurodegeneration, characteristics of Alzheimer disease	overexpression	Microtubule-binding protein	(Wittmann et al., 2001, Jackson et al., 2002)

	Mushroom-body expressed (MUB)	*D. melanogaster*	neurodegeneration, tauopathy	loss of function	RNA-processing	(Park et al., 2004, Shulman and Feany, 2003)
	p53 (short isoform with missing 5' end)	*M. musculus*	shortened life span, increased cancer/ decreased apoptosis, highest expression during early embryogenesis	overexpression	p53 is a tumor suppressor/DNA-binding transcription factor important for apoptosis	(Maier et al., 2004)
	PASG	*M. musculus*	Homozygotes died after max. 25 days	loss of function	Proliferation associated SNF2-like gene (Helicase that regulates chromatin remodelling for methylation)	(Sun et al., 2004)
	PDE2	*S. cerevisiae*	Shortened life span by 75%	loss of function	Phosphodiesterase that catalyzes the hydrolysis of cAMP	(Lin et al., 2000, Wilson and Tatchell, 1988)
	POL1	*S. cerevisiae*	shortened life span by 20-60%	loss of function	DNA polymerase I alpha subunit p180	(Hoopes et al., 2002)

	PolgA	*M. musculus*	Reduced life span, premature aging (e.g. osteoporosis, reduced fertility, weight loss)	loss of function	Nucleus-encoded catalytic subunit of mitochondrial DNA polymerase	(Trifunovic et al., 2004)
	RAD27	*S. cerevisiae*	shortened life span by 60%	loss of function	5' to 3' exonuclease required for Okazaki fragment processing	(Hoopes et al., 2002)
	RAD9	*S. cerevisiae*	shortened life span by 30%	loss of function	Required for DNA-damage arrest in G2 and G1	(Kennedy et al., 1994)
	RAS1	*S. cerevisiae*	shortened stationary phase survival	loss of function	GTP-binding protein (regulation of cAMP pathway)	(Fabrizio and Longo, 2003)
	RAS2	*S. cerevisiae*	shortened life span by 30%	loss of function	GTP-binding protein (regulation of cAMP pathway)	(Sun et al., 1994)
	rgn	*M. musculus*	50% dead after 180days (Maruyama,unpublished)	loss of function	Senescence marker protein-30 (protect cells from aging)	(Ishigami et al., 2002)
	RIF1	*S. cerevisiae*	shortened life span by 40%, increased telomeric silencing and telomere length	loss of function	Unknown, interacts with Rap1p	(Austriaco and Guarente, 1997, Hardy et al., 1992)
	SGS1	*S. cerevisiae*	shortened life span by 60%, progressive sterility, fragmentation of the nucleolus, movement of the SIR complex to the nucleolus	loss of function	DNA helicase with 3'-5' polarity	(Bennett et al., 1998, Sinclair et al., 1997)

	SIM1	*S. cerevisiae*	shortens mean life span, cells are unable to undergo mitosis and arrest in G2	loss of function	unknown	(Austriaco, 1996)
	SIR2	*S. cerevisiae*	Shortened life span by 50%, increased formation of extrachromosomal rDNA circles	loss of function	NAD-dependent histone deacetylase, ADP-ribosyltransferase	(Tanny et al., 1999)
	SIR3/SIR4	*S. cerevisiae*	shortened life span by 20-25%	loss of function	Silencing factor	(Kaeberlein et al., 1999)
	SNF1	*S. cerevisiae*	shortened life span by 25%	overexpression	Serine/threonine kinase, required to derepress genes	(Ashrafi et al., 2000)
	Srs-2	*S. cerevisiae*	shortens life span up to 60% in yeast	loss of function	DNA helicase	(McVey et al., 2001)
	SUN4	*S. cerevisiae*	shortened life span by ~12.5%	loss of function	cell septation	(Austriaco, 1996, Camougrand et al., 2000)
	SW14	*S. cerevisiae*	shortened life span by 90%	loss of function	SBF tarnscription factor involved in regulating G1 cyclins and cell wall stability	(Kaeberlein and Guarente, 2002)

	Terc	*M. musculus*	shortened life span by 25%, shortened life span with succeeding generations, hair loss,	loss of function	RNA component of telomerase	(Rudolph et al., 1999)
	Top3b	*M. musculus*	70% shortened life span (if homozygous)	loss of function	Type I topoisomerase	(Kwan and Wang, 2001)
	TRp63	*M. musculus*	21.5% shortened life span (if heterozygous)	loss of function	transcriptional activator and repressor	(Keyes et al., 2005)
	WRN	*H. sapiens*	Werner Syndrome, premature aged facies, cataracts, arteriosclerosis, Diabetes Mellitus	loss of function	DNA helicase (with exonuclease domain)	(Yu et al., 1996)
	XPD	*M. musculus*	life span of 7 months (if homozygous), premature ageing (osteoporosis, infertility)	loss of function	Helicase subunit of TFIIH (DNA repair/ transcription)	(de Boer et al., 2002)
protein degradation	clk-1	*C. elegans*	shortened life span, increased mitochondrial activity	overexpression	required for ubiquinone biosynthesis	(Felkai et al., 1999)
	COQ3	*S. cerevisiae*	shortened life span	loss of function	Hexaprenyldihydroxybenzoate methyltransferase, involved in ubiquinone metabolism	(Fabrizio and Longo, 2003, Fabrizio et al., 2003)

	Ubiquilin or *ubiquitin C-terminal hydrolase*	*H. sapiens*	Parkinson's disease, cancer	loss of function	Protein degradation	(Leroy et al., 1998, Maraganore et al., 1999, Li et al., 2007)
	PHB1/PHB2	*S. cerevisiae*	shortened life span by 20%, ageing of cells (lengthening of cell cycle), deletion in both results in 40% reduced life span	loss of function	Phb1p/Phb2p complex inhibits degradation of proteins in the mitochondrial membrane	(Coates et al., 1997)
protein regulation	aak-2	*C. elegans*	shortens life span by 12%	loss of function	AMP-activated protein kinase	(Apfeld et al., 2004)
	BCY1	*S. cerevisiae*	shortened life span	loss of function	Regulatory subunit of protein kinase A (PKA)	(Sun et al., 1994, Fabrizio et al., 2003)
detoxification	CTT1	*S. cerevisiae*	shortened life span	overexpression	Cytosolic catalase T	(Fabrizio et al., 2003)
	glutathione transferase	*D.melanogaster*	reduced life span	loss of function	cell protection for oxidative damage	(Toba and Aigaki, 2000)
	hsf-1	*C. elegans*	shortened life span by 35%	RNAi	Transcription factor that regulates response to heat and oxidative stress	(Garigan et al., 2002)

	Prdx1(Pag or MSP23)	*M. musculus*	shortened life span, anaemia, malignant cancer	loss of function	Peroxiredoxin (Antioxidant enzyme that scavenges peroxides and uses thioredoxin as electron donor)	(Neumann et al., 2003)
	SOD1	*S. cerevisae*	shortened chronological/ replicative life span	loss of function	cytosolic copper-zinc superoxide dismutase	(Longo et al., 1999)
	SOD2	*S. cerevisiae*	decreased survival in stationary phase	loss of function	Mn- superoxide dismutase of the mitochondrial matrix	(Longo et al., 1999)
	SOD2	*M. musculus*	small, pale, die within 7-10 days	loss of function	Mn- superoxide dismutase	(Li et al., 1995)
	thioredoxin-1	*C. elegans* *M. musculus*	life span decrease (*C. elegans*), in mice lethal (trx-2?)	loss of function	peroxide elimination, protein thiol/ disulfide regulation and as coenzymes + basics for transition between proliferation, differentiation and apoptosis	(Miranda-Vizuete et al., 2006)
	Trehalose biosynthesis related proteins e.g. *trehalose-6-phosphate synthetase* (TPS-	*M. musculus*	not thermotolerant	loss of function	cryoprotective, protection for desiccation, stabilise proteins (reduces polyglutamine toxicity), suppress polyglutamine-induced protein aggregation	(Tanaka et al., 2004)

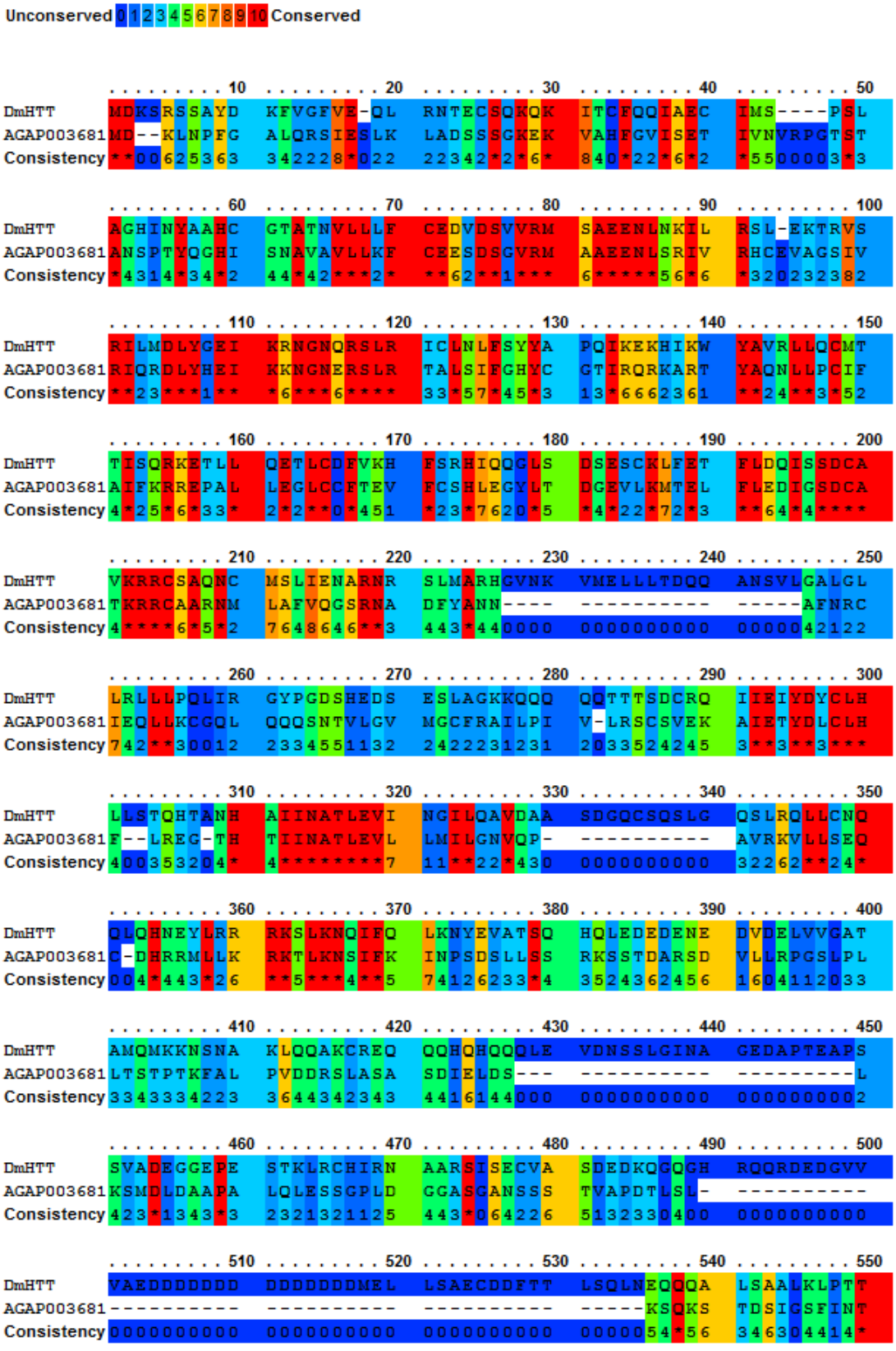
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ATP/NAD synthesis	ATP2	*S. cerevisiae*	shortened life span	loss of function	Beta subunit of the mitochondrial F1-F0 ATPase	(Fabrizio et al., 2003)
	NPT1	*S.cerevisiae*	shortened life span, loss of telomere and rDNA silencing due to loss of SIR2 activity	loss of function	Nicotinate phosphoribosyltransferase, involved in NAD biosynthesis	(Lin et al., 2000)
vesicle transport	beach1	*D. melanogaster*	40-50% reduced life span, reduced CNS size/morphology, accumulation of insoluble ubiquitinated proteins and amyloid precursor-lie proteins, increased neuronal apoptosis	loss of function	intracellular protein transport, lysosomal transport	(Finley et al., 2003)
	Blue cheese (BCHS)	*D. melanogaster*	protein aggregates, neurodegeneration	loss of function	vesicle transport, protein processing	(Finley et al., 2003)
	human polyglutamine repeats (e.g. in huntingtin (HTT), ataxin or as peptides)	*D. melanogaster*	>35 repeats lead to neurodegeneration, onset/ time of death depends on length of repeats	overexpression	unknown for HTT (maybe vesicle/organelle transport; form cation channels when applied to artificial bilayer membrane	(Warrick et al., 1999, Jackson et al., 1998)

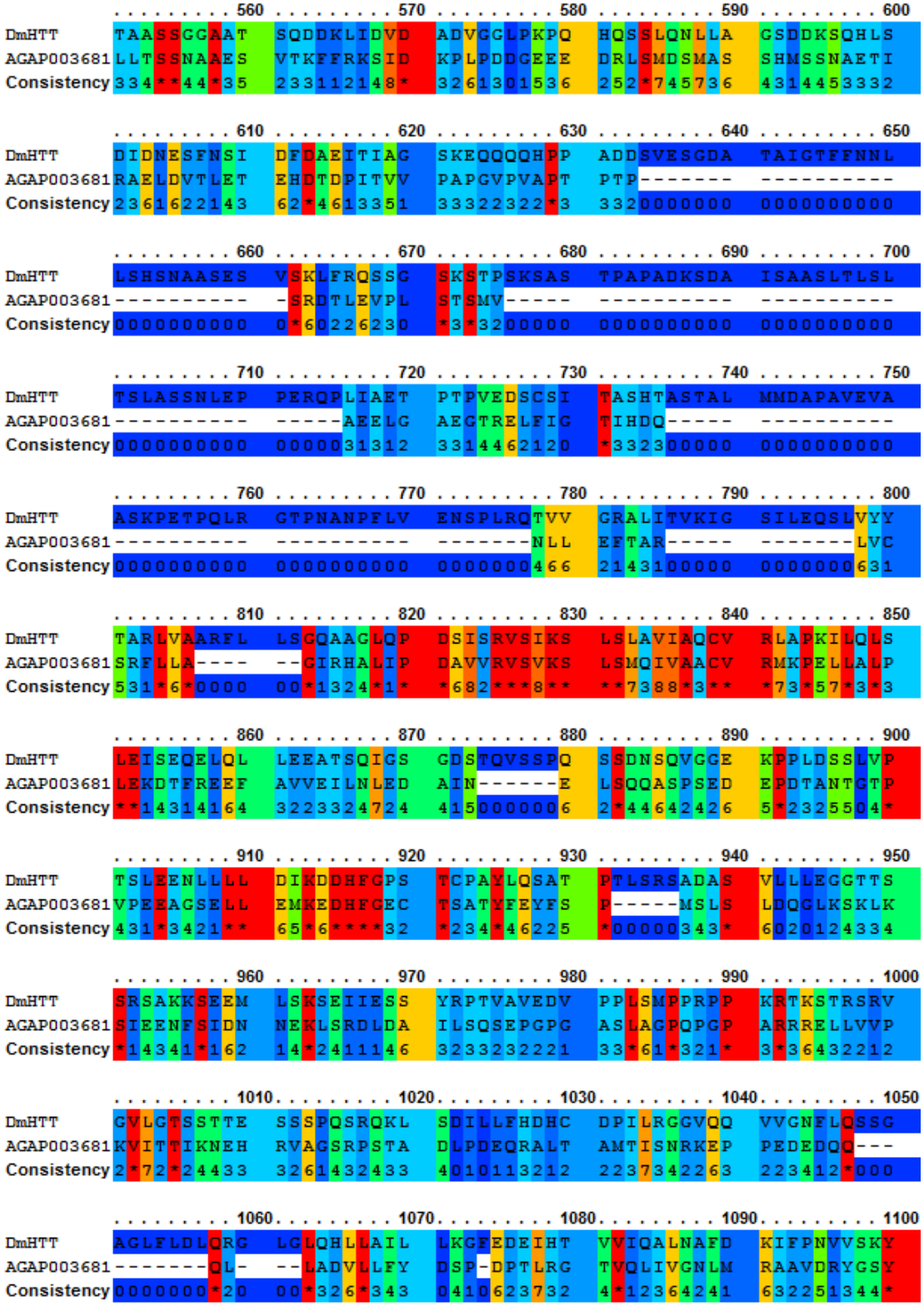
	human alpha-synuclein	*D. melanogaster*	Parkinson (using human alpha synuclein), neurodegeneration	overexpression	vesicle transport	(Feany and Bender, 2000)
Lipid storage	*bubblegum*	*D. melanogaster*	Adenoleukodystrophy (ALD)	loss of function	VLCFA acyl coenzyme A synthetase	(Min and Benzer, 1999)
	Glial Lazarillo (Glaz)	*D. melanogaster*	loss of function mutations in GLaz shortened life-span and decreased resistance to oxidative stress	loss of function	Apolipoprotein D	(Sanchez et al., 2006)
	GPD1	*S. cerevisiae*	shortened life span by 25%	loss of function	Glycerol-3-phosphate dehydrogenase	(Kaeberlein et al., 2002)
	4-hydroxy-2-nonenal (HNE) cross-linked protein		Lipofuscin accumulation , destroys lysosomal integrity	overexpression	HNE- lipid peroxidation product, proteosome inhibition	(Terman and Sandberg, 2002)
	pnk-1	*C. elegans*	shortened life span, reduced lipid	RNAi	Pantothenate kinase involved in	(Lee et al., 2003b, Lin et

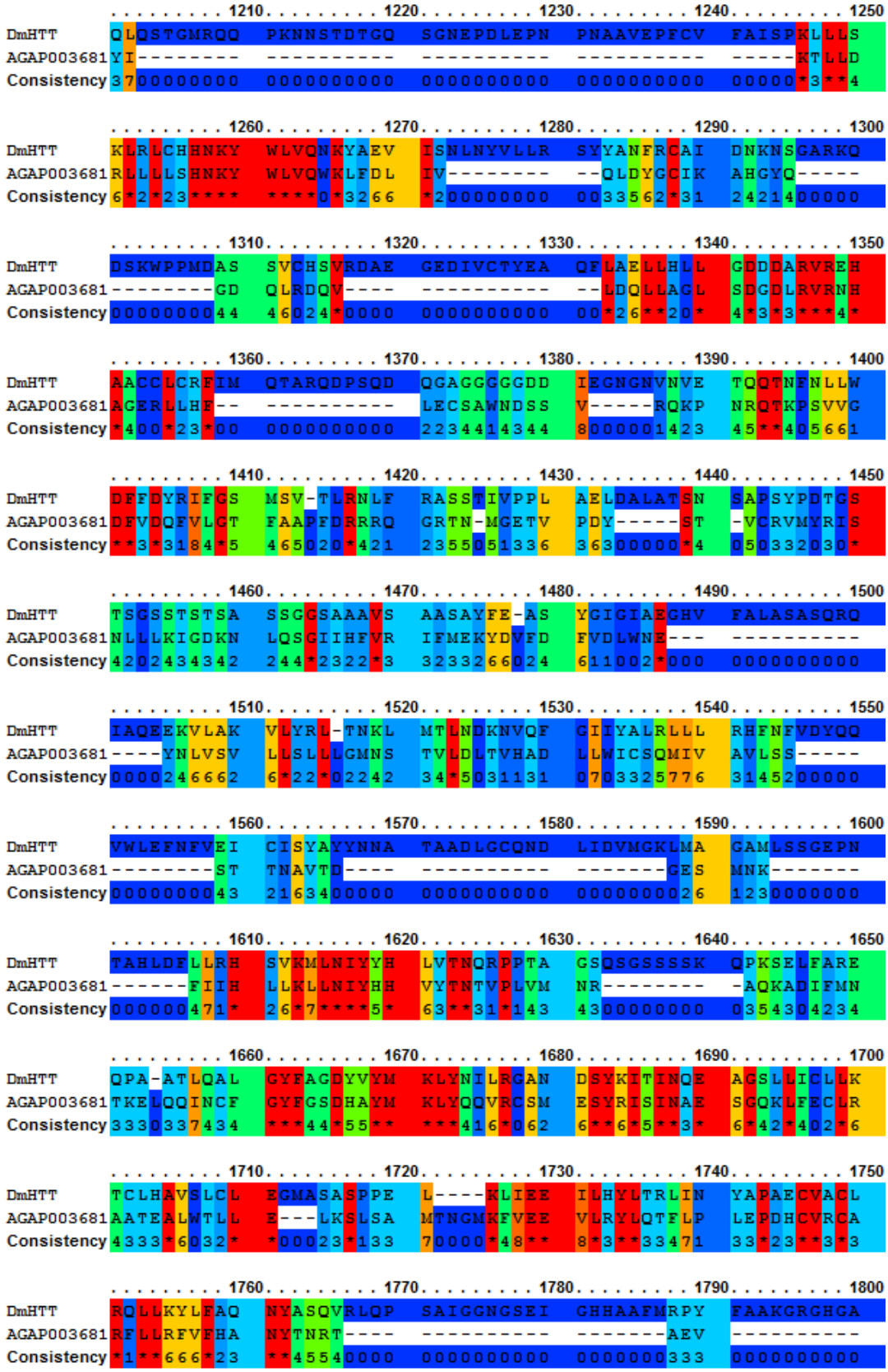
			storage		coenzyme A biosynthesis	al., 1997)
Insulin-signalling	daf-16	*C. elegans*	some alleles have shortened life span	loss of function	Forkhead transcription factor	(Lin et al., 1997, Clancy et al., 2001)
	PKB	*D. melanogaster*	decreased life span	loss of function	Protein kinase B, age-1 homolog	(Clancy et al., 2001)
others	AdA-1	*S. cerevisiae*	shortens life span by 80%, no premature ageing phenotype	loss of function	transcription factor in ADA complex	(Sinclair et al., 1997, Kaeberlein and Guarente, 2002)
	CCR4	*S.cerevisiae*	shortened life span by 80%	loss of function	transcription factor involved in non-fermentative growth and cell wall stability	(Kaeberlein and Guarente, 2002)
	eat-7	*C. elegans*	decreased life span by 35%, defects in pharyngeal feeding behaviour	loss of function	unknown	(Lakowski and Hekimi, 1998)
	eggroll	*D. melanogaster*	dense, multilamellated structures (~Tay-Sachs), not temp sensitive, reduced life span, x-linked recessive	loss of function	unknown	(Min and Benzer, 1997a)
	GH-1	*H. sapiens*	shortened life span, dwarfism	loss of function	Human growth hormone	(Besson et al.,

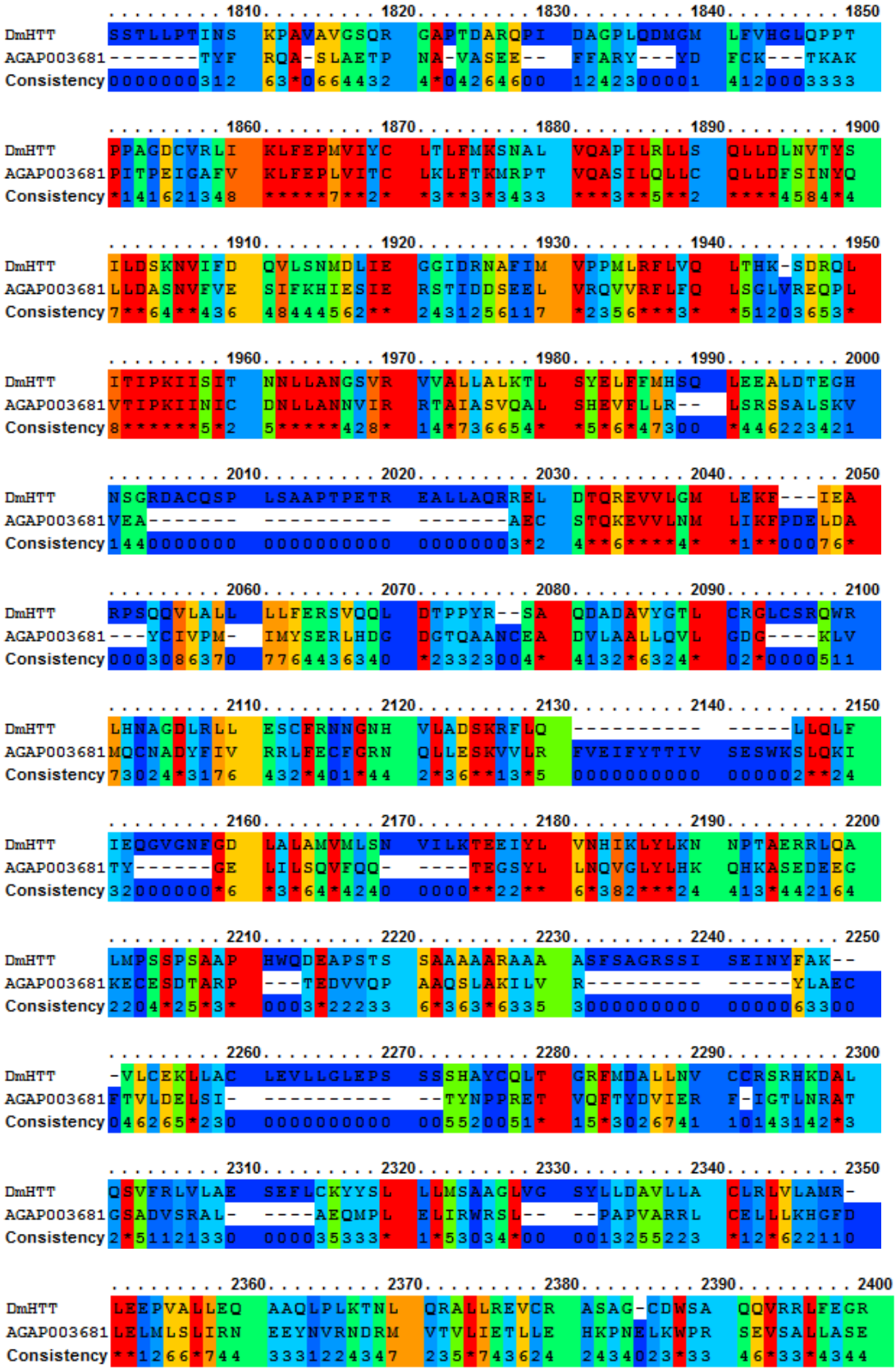
						2003)
	HAP5	*S. cerevisiae*	shortened life span by 40%	loss of function	HAP complex transcription factor	(Sinclair et al., 1997)
	Hutchinson-Gilford Syndrome (LMNA)	*H. sapiens*	die at ~ age of 10 of coronary heart disease, prematurely senile appearing skin/hair	loss of function	components of nuclear lamina	(Beard et al., 2008)
	Klotho	*M. musculus*	life span ~60 days, growth retardation at 3-4 weeks	loss of function	Beta-glucosidase	(Kuroo et al., 1997)
	MATa/alpha	*S.cerevisiae*	20% reduced life span, mating-type heterozygosity	loss of function	N/A	(Kaeberlein et al., 1999, Kaeberlein and Guarente, 2002)
	MPT5	*S.cerevisiae*	reduced life span up to 50%, temperature sensitive	loss of function	post-transcriptional repressor of HO	(Kaeberlein and Guarente, 2002)
	nhr-49	*C. elegans*	30-40% reduced life span, accumulation of fat	RNAi	Nuclear hormone receptor	(Van Gilst et al., 2005)
	RSR1	*S. cerevisiae*	shortened life span	loss of function	GTPase involved in bud site selection	(Jazwinski et al., 1998)

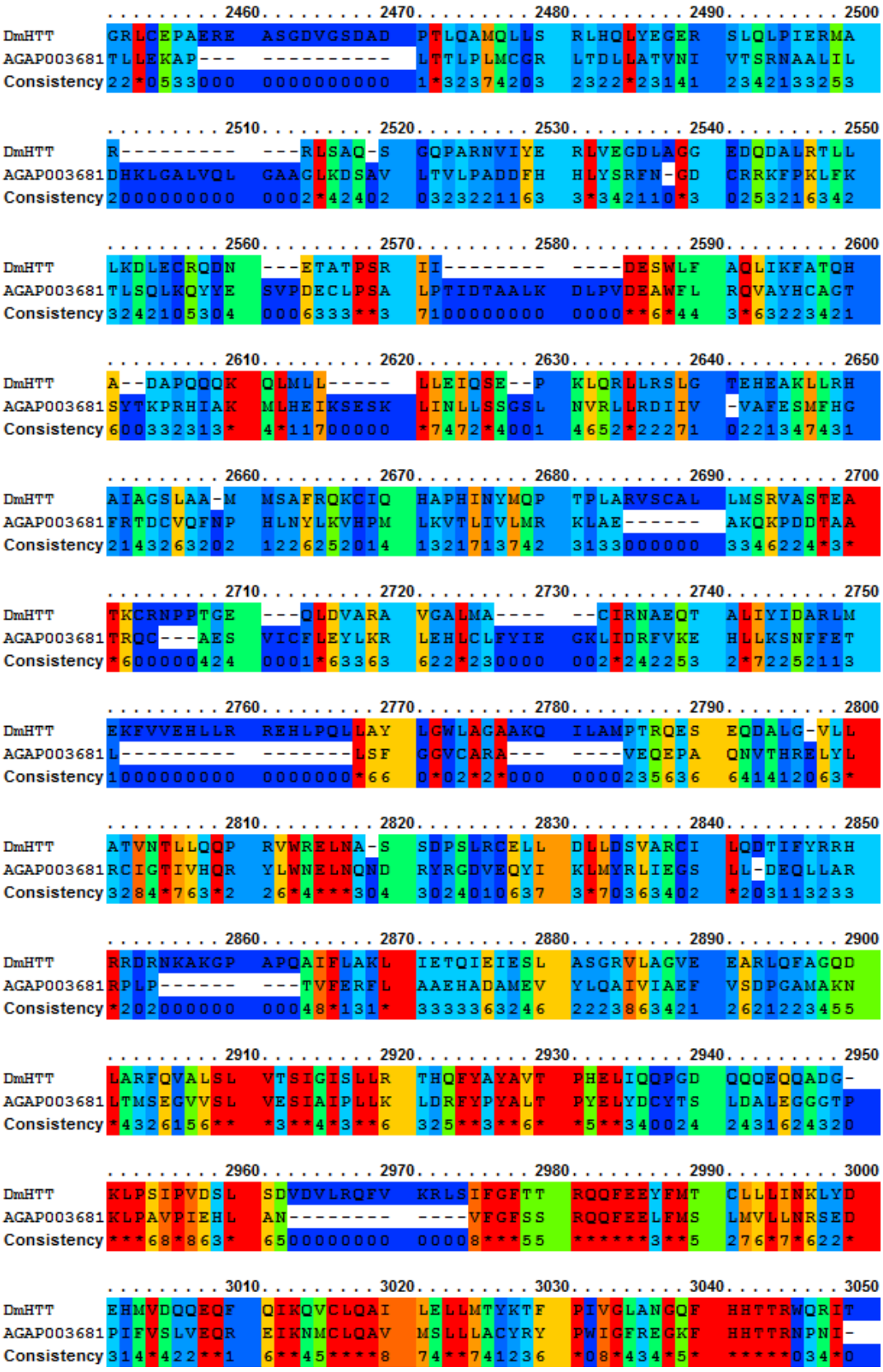
	RTG2	*S. cerevisiae*	shortened life span	loss of function	transcription factor necessary for the retrograde response	(Liao and Butow, 1993)
	scl-1	*C. elegans*	slight decrease in life span	RNAi	unknown, putative secretory protein	(Ookuma et al., 2003)
	SIP1/SIP2	*S. cerevisae*	20-40% reduced life span	loss of function	protein involved in glucose repression, possibly adapters to direct Snf1p activity	(Ashrafi et al., 2000)
	spongecake	*D. melanogaster*	membrane-bound vesicles (Creutzfeld Jacob) neurodegeneration, but heterozygous normal life, phenotype: temperature sensitive (no life span decrease at 25ºC), x-linked recessive	loss of function	unknown	(Min and Benzer, 1997a)
	ZDS2	*S. cerevisiae*	20% reduced life span	loss of function	unknown	(Roy and Runge, 2000)

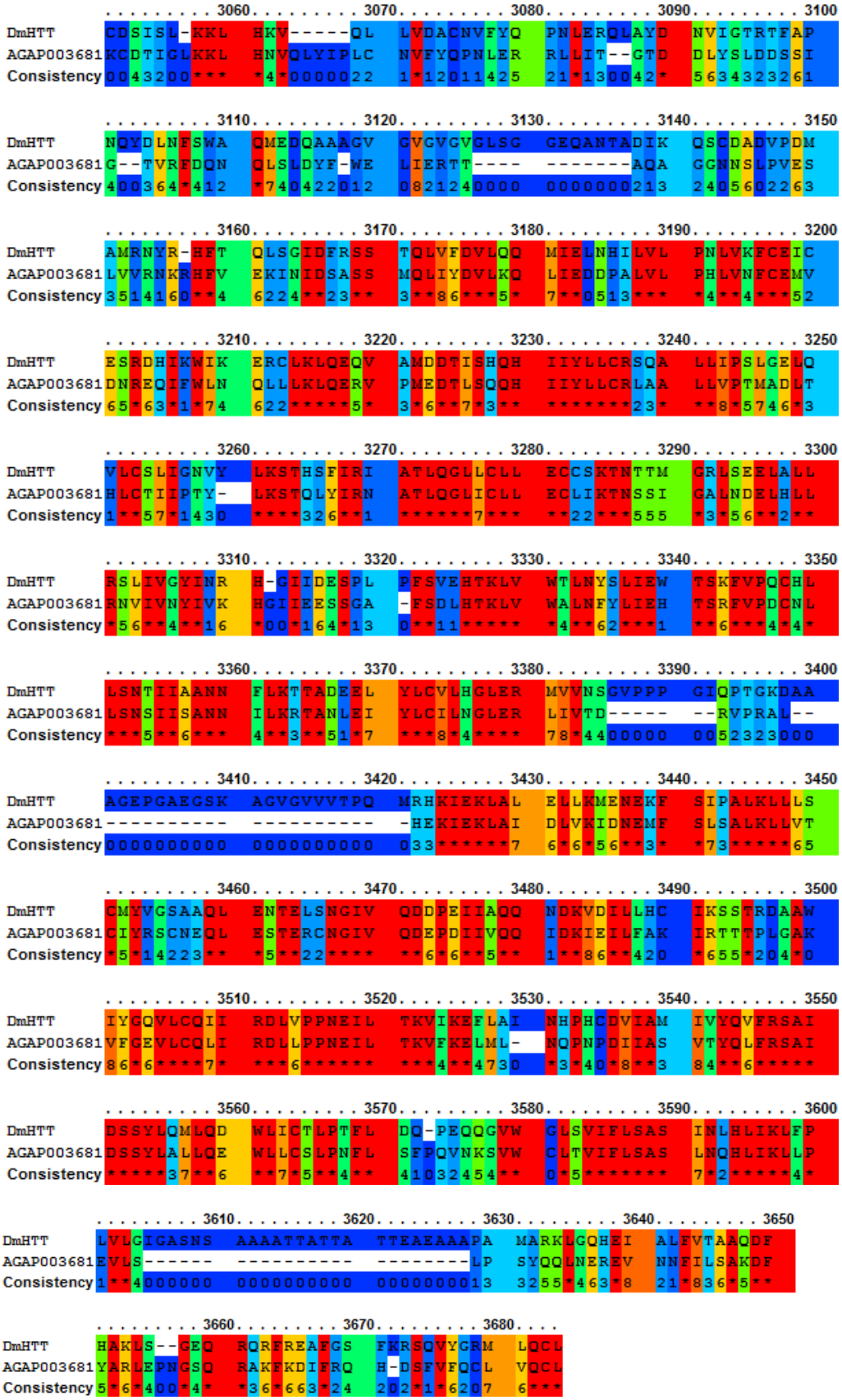

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Consistency


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DmHTT ASKPETPQLR GTPNANPFLV ENSPLRQTVV GRALITVKIG SILEQSLVYY
AGAP003681 ---------- ---------- -------NLL EFTAR----- -------LVC
Consistency 0000000000 0000000000 0000000466 2143100000 0000000631
810 820 830 840 850
DmHTT TARLVAARFL LSGQAAGLQP DSISRVSIKS LSLAVIAQCV RLAPKILQLS
AGAP003681 SRFLLA---- --GIRHALIP DAVVRVSVKS LSMQIVAACV RMKPELLALP
Consistency 531*6*0000 00*1324*1* *682***8** **7388*3** *73*57*3*3
860 870 880 890 900
DmHTT LEISEQELQL LEEATSQIGS GDSTQVSSPQ SSDNSQVGGE KPPLDSSLVP
AGAP003681 LEKDTFREEF AVVEILNLED AIN------E LSQQASPSED EPDTANTGTP
Consistency **143141 64 322332472 4 415000000 6 2*44642426 5*232550 4*
910 920 930 940 950
DmHTT TSLEENLLLL DIKDDHFGPS TCPAYLQSAT PTLSRSADAS VLLLEGGTTS
AGAP003681 VPEEAGSELL EMKEDHFGEC TSATYFEYFS P-----MSLS LDQGLKSKLK
Consistency 431*3421** 65*6**** 32 *234*46225 *00000343* 6020124334
960 970 980 990 1000
DmHTT SRSAKKSEEM LSKSEIIESS YRPTVAVEDV PPLSMPPRPP KRTKSTRSRV
AGAP003681 SIEENFSIDN NEKLSRDLDA ILSQSEPGPG ASLAGPQPGP ARRRELLVVP
Consistency *14341*162 14*2411146 3233232221 33*61*321* 3*36432212
1010 1020 1030 1040 1050
DmHTT GVLGTSSTTE SSSPQSRQKL SDILLFHDHC DPILRGGVQQ VVGNFLQSSG
AGAP003681 KVITTIKNEH RVAGSRPSTA DLPDEQRALT AMTISNRKEP PEDEDQQ---
Consistency 2*72*24433 32614324 33 4010113212 2237342263 223412*000
1060 1070 1080 1090 1100
DmHTT AGLFLDLQRG LGLQHLLAIL LKGEEDEIHT VVIQALNAFD KIFPNVVSKY
AGAP003681 -------QL- --LADVLLFY DSP-DPTLRG TVQLIVGNLM RAAVDRYGSY
Consistency 0000000*20 00*326*343 0410623732 4*1236424 1 6322513 44*

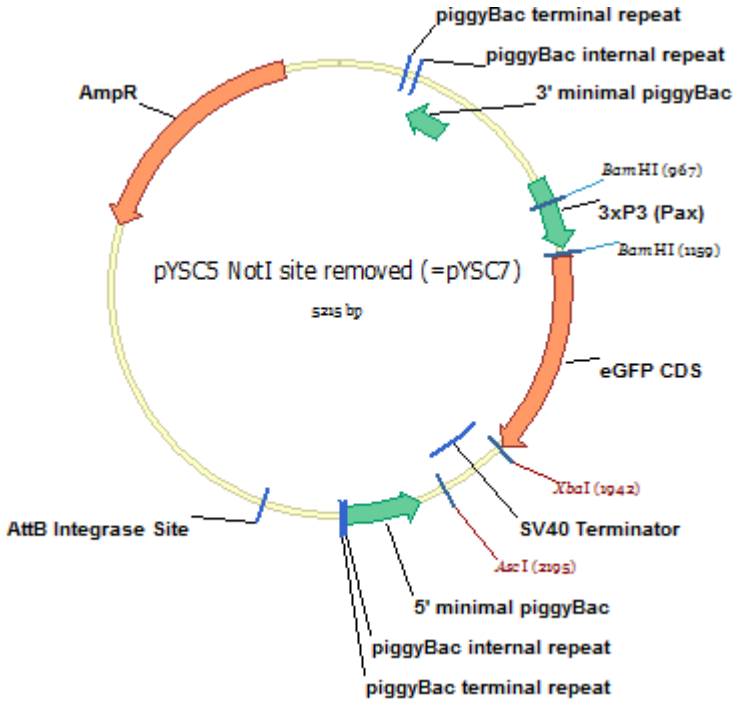

1210 1220 1230 1240 1250
DmHTT QLQSTGMRQQ PKNNSTDTGQ SGNEPDLEPN PNAAVEPFCV FAISPKLLLS
AGAP003681 YI--------- ---------- ---------- ---------- -----KTLLD
Consistency 370000000000 0000000000 0000000000 0000000000 00000*3**4
1260 1270 1280 1290 1300
DmHTT KLRLCHHNKY WLVQNKYAEV ISNLNYVLLR SYYANFRCAI DNKNSGARKQ
AGAP003681 RLLLLSHNKY WLVQWKLFDL IV-------- --QLDYGCIK AHGYQ-----
Consistency 6*2*23**** ****0*3266 *200000000 0033562*31 2421400000
1310 1320 1330 1340 1350
DmHTT DSKWPPMDAS SVCHSVRDAE GEDIVCTYEA QFLAELLHLL GDDDARVREH
AGAP003681 --------GD QLRDQV---- ---------- --LDQLLAGL SDGDLRVRNH
Consistency 00000000044 4602 4*0000 0000000000 00*26**20* 4*3*3***4*
1360 1370 1380 1390 1400
DmHTT AACCLCRFIM QTARQDPSQD QGAGGGGGDD IEGNGNVNVE TQQTNFNLLW
AGAP003681 AGERLLHF-- ---------- LECSAWNDSS V-----RQKP NRQTKPSVVG
Consistency *400*23*00 0000000000 2234414344 8000001423 45**405661
1410 1420 1430 1440 1450
DmHTT DFFDYRIFGS MSV-TLRNLF RASSTIVPPL AELDALATSN SAPSYPDTGS
AGAP003681 DFVDQFVLGT FAAPFDRRRQ GRTN-MGETV PDY-----ST -VCRVMYRIS
Consistency **3*3184*5 4650 20*421 2355051336 3630 0000*4 0503320 30*
1460 1470 1480 1490 1500
DmHTT TSGSSTSTSA SSGGSAAAVS AASAYFE-AS YGIGIAEGHV FALASASQRQ
AGAP003681 NLLLKIGDKN LQSGIIHFVR IFMEKYDVFD FVDLWNE--- ----------
Consistency 420243434 2 244*2322*3 3233266024 611002*000 0000000000
1510 1520 1530 1540 1550
DmHTT IAQEEKVLAK VLYRL-TNKL MTLNDKNVQF GIIYALRLLL RHFNFVDYQQ
AGAP003681 ----YNLVSV LLSLLLGMNS TVLDLTVHAD LLWICSQMIV AVLSS-----
Consistency 0000246662 6*22*02242 34*5031131 0703325776 3145200000
1560 1570 1580 1590 1600
DmHTT VWLEFNFVEI CISYAYYNNA TAADLGCQND LIDVMGKLMA GAMLSSGEPN
AGAP003681 --------ST TNAVTD---- ---------- -------GES MNK-------
Consistency 00000000043 2163400000 0000000000 0000000026 1230000000
1610 1620 1630 1640 1650
DmHTT TAHLDFLLRH SVKMLNIYYH LVTNQRPPTA GSQSGSSSSK QPKSELFARE
AGAP003681 ------FIIH LLKLLNIYHH VYTNTVPLVM NR-------- -AQKADIFMN
Consistency 000000471* 26*7****5* 63**31*143 4300000000 0354304234
1660 1670 1680 1690 1700
DmHTT QPA-ATLQAL GYFAGDYVYM KLYNILRGAN DSYKITINQE AGSLLICLLK
AGAP003681 TKELQQINCF GYFGSDHAYM KLYQQVRCSM ESYRISINAE SGQKLFECLR
Consistency 3330337434 ***44*55** ***416*062 6**6*5**3* 6*42*402*6
1710 1720 1730 1740 1750
DmHTT TCLHAVSLCL EGMASASPPE L----KLIEE ILHYLTRLIN YAPAECVACL
AGAP003681 AATEALWTLL E---LKSLSA MTNGMKFVEE VLRYLQTFLP LEPDHCVRCA
Consistency 4333*6032* *000 23*133 70000*48** 8*3**33471 33*23**3*3
1760 1770 1780 1790 1800
DmHTT RQLLKYLFAQ NYASQVRLQP SAIGGNGSEI GHHAAFMRPY FAAKGRGHGA
AGAP003681 RFLLRFVFHA NYTNRT---- ---------- -------AEV ----------
Consistency *1**666*23 **4554 0000 0000000000 0000000333 0000000000


1810 1820 1830 1840 1850
DmHTT SSTLLPTINS KPAVAVGSQR GAPTDARQPI DAGPLQDMGM LFVHGLQPPT
AGAP003681 -------TYF RQA-SLAETP NA-VASEE-- FFARY---YD FCK---TKAK
Consistency 0000000312 63*0664432 4*042646 00 1242300001 4120003333
1860 1870 1880 1890 1900
DmHTT PPAGDCVRLI KLFEPMVIYC LTLFMKSNAL VQAPILRLLS QLLDLNVTYS
AGAP003681 PITPEIGAFV KLFEPLVITC LKLFTKMRPT VQASILQLLC QLLDFSINYQ
Consistency *14162134 8 *****7**2* *3**3*3433 ***3**5**2 ****4584*4
1910 1920 1930 1940 1950
DmHTT ILDSKNVIFD QVLSNMDLIE GGIDRNAFIM VPPMLRFLVQ LTHK-SDRQL
AGAP003681 LLDASNVFVE SIFKHIESIE RSTIDDSEEL VRQVVRFLFQ LSGLVREQPL
Consistency 7**64**436 484445 62** 2431256117 *2356***3* *5120365 3*
1960 1970 1980 1990 2000
DmHTT ITIPKIISIT NNLLANGSVR VVALLALKTL SYELFFMHSQ LEEALDTEGH
AGAP003681 VTIPKIINIC DNLLANNVIR RTAIASVQAL SHEVFLLR-- LSRSSALSKV
Consistency 8******5*2 5*****428* 14*736654* *5*6*47300 *4462234 21
2010 2020 2030 2040 2050
DmHTT NSGRDACQSP LSAAPTPETR EALLAQRREL DTQREVVLGM LEKF---IEA
AGAP003681 VEA------- ---------- -------AEC STQKEVVLNM LIKFPDELDA
Consistency 144000000 0 0000000000 0000000 3*2 4**6****4* *1**000 76*
2060 2070 2080 2090 2100
DmHTT RPSQQVLALL LLFERSVQQL DTPPYR--SA QDADAVYGTL CRGLCSRQWR
AGAP003681 ---YCIVPM- IMYSERLHDG DGTQAANCEA DVLAALLQVL GDG----KLV
Consistency 000308637 0 776443634 0 *2332300 4* 4132*6324* 02*0000 511
2110 2120 2130 2140 2150
DmHTT LHNAGDLRLL ESCFRNNGNH VLADSKRFLQ ---------- -----LLQLF
AGAP003681 MQCNADYFIV RRLFECFGRN QLLESKVVLR FVEIFYTTIV SESWKSLQKI
Consistency 7302 4*3176 432*401*44 2*36**13*5 0000000000 000002**24
2160 2170 2180 2190 2200
DmHTT IEQGVGNFGD LALAMVMLSN VILKTEEIYL VNHIKLYLKN NPTAERRLQA
AGAP003681 TY------GE LILSQVFQQ- ----TEGSYL LNQVGLYLHK QHKASEDEEG
Consistency 32000000*6 *3*64*4240 0000**22** 6*382***24 413*442164
2210 2220 2230 2240 2250
DmHTT LMPSSPSAAP HWQDEAPSTS SAAAAARAAA ASFSAGRSSI SEINYFAK--
AGAP003681 KECESDTARP ---TEDVVQP AAQSLAKILV R--------- -----YLAEC
Consistency 2204*25*3* 0003*22233 6*363*6335 3000000000 00000633 00
2260 2270 2280 2290 2300
DmHTT -VLCEKLLAC LEVLLGLEPS SSSHAYCQLT GRFMDALLNV CCRSRHKDAL
AGAP003681 FTVLDELSI- ---------- --TYNPPRET VQFTYDVIER F-IGTLNRAT
Consistency 0462 65*230 0000000000 0055200 51* 15*3026741 1014314 2*3
2310 2320 2330 2340 2350
DmHTT QSVFRLVLAE SEFLCKYYSL LLMSAAGLVG SYLLDAVLLA CLRLVLAMR-
AGAP003681 GSADVSRAL- ----AEQMPL ELIRWRSL-- --PAPVARRL CELLLKHGFD
Consistency 2*5112133 0 0000 35333* 1*53034*00 0013255223 *12*622110
2360 2370 2380 2390 2400
DmHTT LEEPVALLEQ AAQLPLKTNL QRALLREVCR ASAG-CDWSA QQVRRLFEGR
AGAP003681 LELMLSLIRN EEYNVRNDRM VTVLIETLLE HKPNELKWPR SEVSALLASE
Consistency **1266*744 3331224347 235*743624 2434023*33 46*33*4344

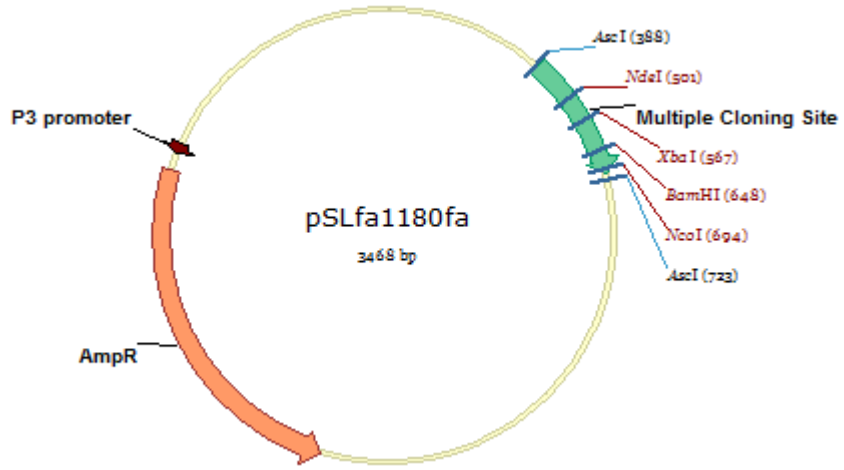

2460 2470 2480 2490 2500
DmHTT GRLCEPAERE ASGDVGSDAD PTLQAMQLLS RLHQLYEGER SLQLPIERMA
AGAP003681 TLLEKAP--- ---------- LTTLPLMCGR LTDLLATVNI VTSRNAALIL
Consistency 22*0533000 0000000000 1*3237420 3 2322*23141 2342133253
2510 2520 2530 2540 2550
DmHTT R--------- ---RLSAQ-S GQPARNVIYE RLVEGDLAGG EDQDALRTLL
AGAP003681 DHKLGALVQL GAAGLKDSAV LTVLPADDFH HLYSRFN-GD CRRKFPKLFK
Consistency 2000000000 0002*42402 0323221163 3*3421102*3 0253216342
2560 2570 2580 2590 2600
DmHTT LKDLECRQDN ---ETATPSR II-------- ----DESWLF AQLIKFATQH
AGAP003681 TLSQLKQYYE SVPDECLPSA LPTIDTAALK DLPVDEAWFL RQVAYHCAGT
Consistency 3242105304 0006333**3 7100000000 0000**6*44 3*63223421
2610 2620 2630 2640 2650
DmHTT A--DAPQQQK QLMLL----- LLEIQSE--P KLQRLLRSLG TEHEAKLLRH
AGAP003681 SYTKPRHIAK MLHEIKSESK LINLLSSGSL NVRLLRDIIV -VAFESMFHG
Consistency 600332313* 4*1170000 *7472*4001 4652*22271 0221347431
2660 2670 2680 2690 2700
DmHTT AIAGSLAA-M MSAFRQKCIQ HAPHINYMQP TPLARVSCAL LMSRVASTEA
AGAP003681 FRTDCVQFNP HLNYLKVHPM LKVTLIVLMR KLAE------ AKQKPDDTAA
Consistency 2143263202 1226252014 1321713742 3133000000 3346224*3*
2710 2720 2730 2740 2750
DmHTT TKCRNPPTGE ---QLDVARA VGALMA---- --CIRNAEQT ALIYIDARLM
AGAP003681 TRQC---AES VICFLEYLKR LEHLCLFYIE GKLIDRFVKE HLLKSNFFET
Consistency *600000424 0001*63363 622*230000 002*242253 2*7225211 3
2760 2770 2780 2790 2800
DmHTT EKFVVEHLLR REHLPQLLAY LGWLAGAAKQ ILAMPTRQES EQDALG-VLL
AGAP003681 L--------- -------LSF GGVCARA--- ----VEQEPA QNVTHRELYL
Consistency 1000000000 0000000*66 0*02*2*000 00002356 36 6414120 63*
2810 2820 2830 2840 2850
DmHTT ATVNTLLQQP RVWRELNA-S SDPSLRCELL DLLDSVARCI LQDTIFYRRH
AGAP003681 RCIGTIVHQR YLWNELNQND RYRGDVEQYI KLMYRLIEGS LL-DEQLLAR
Consistency 3284*763*2 26*4***304 3024010637 3*7036340 2 *203113233
2860 2870 2880 2890 2900
DmHTT RRDRNKAKGP APQAIFLAKL IETQIEIESL ASGRVLAGVE EARLQFAGQD
AGAP003681 RPLP------ ---TVFERFL AAEHADAMEV YLQAIVIAEF VSDPGAMAKN
Consistency *202000000 00048*131* 3333363246 2223863421 2621223455
2910 2920 2930 2940 2950
DmHTT LARFQVALSL VTSIGISLLR THQFYAYAVT PHELIQQPGD QQQEQQADG-
AGAP003681 LTMSEGVVSL VESIAIPLLK LDRFYPYALT PYELYDCYTS LDALEGGGTP
Consistency *4326156** *3**4*3**6 325**3**6* *5**3400 24 2431624320
2960 2970 2980 2990 3000
DmHTT KLPSIPVDSL SDVDVLRQFV KRLSIFGFTT RQQFEEYFMT CLLLINKLYD
AGAP003681 KLPAVPIEHL AN-------- ----VFGFSS RQQFEELFMS LMVLLNRSED
Consistency ***68*863* 650000000 0000 8***55 ******3**5 276*7*622*
3010 3020 3030 3040 3050
DmHTT EHMVDQQEQF QIKQVCLQAI LELLMTYKTF PIVGLANGQF HHTTRWQRIT
AGAP003681 PIFVSLVEQR EIKNMCLQAV MSLLLACYRY PWIGFREGKF HHTTRNPNI-
Consistency 314*422**1 6**45****8 74**741236 *08*434*5* *****034*0



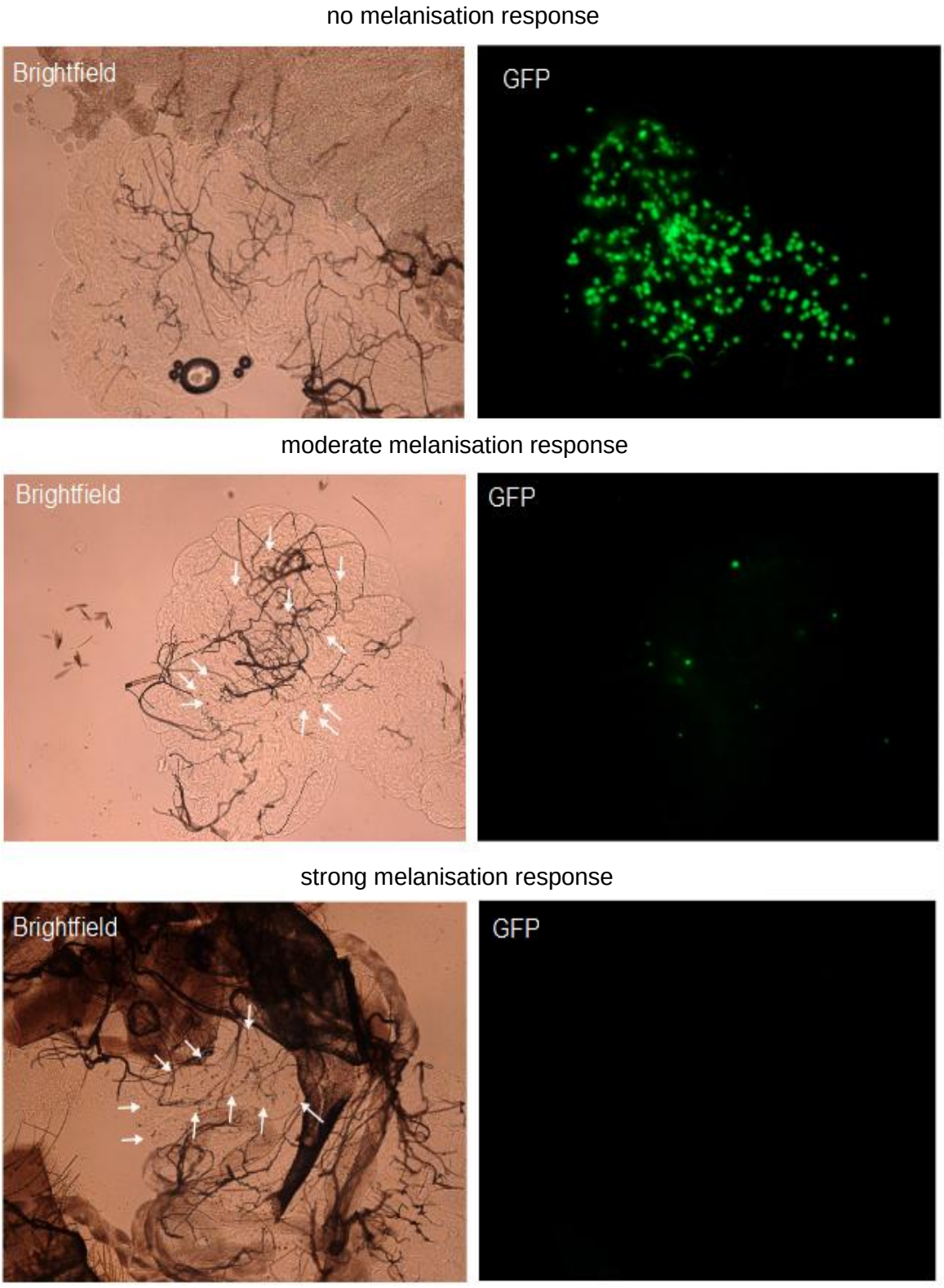
Appendix Figure 10.1: Multiple alignment of the HTT protein sequence of *D. melanogaster* (DmeHn, accession number NP_651629.1) and AGAP003681 of *An. gambiae* (accession number XP_001230941.2) using the software praline (28% sequence identity).



Appendix Figure 10.2: PYSC7 transformation vector (Chan and Windbichler, unpublished).



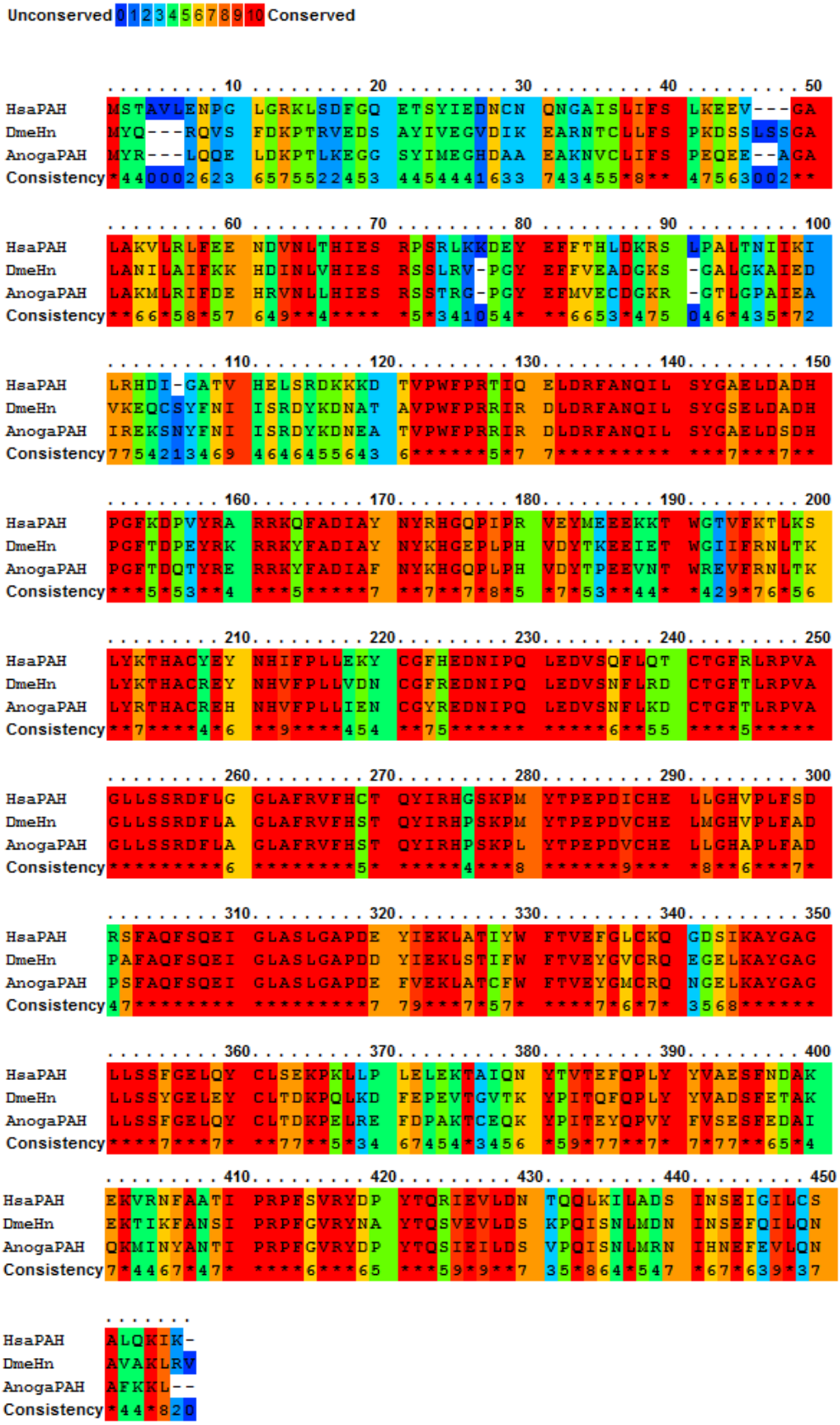
Appendix Figure 10.3: pSLfa1180 shuttle vector.



Appendix Figure 10.4: Displayed are different melanisation responses that can be found in the female An. gambiae midgut 8 days after *P. berghei* infection. The arrows are pointing towards melanised oocysts, the green spots are GFP labelled vital unmelanised ooysts.

Appendix Table 10.2: Identified compounds from two extracted mosquitoes with 80% methanol using GC-MS.

SUPER PATHWAY	SUB PATHWAY	BIOCHEMICAL NAME
Amino acid	Alanine and aspartate metabolism	alanine beta-alanine aspartate N-acetylaspartate (NAA) asparagine
	Glutamate metabolism	glutamate
	Phenylalanine & tyrosine metabolism	phenyllactate (pla) phenylalanine (phe) phenylpyruvate (ppa) tyrosine (tyr)
	Tryptophan metabolism	xanthurenate
	Valine, leucine and isoleucine metabolism	leucine
	cysteine and methionine metabolism	methionine sulfoxide methionine
	Urea cycle; arginine-, proline-, metabolism	4-guanidinobutyrate ornithine uric acid proline
	Polyamine metabolism	putrescine
Carbohydrate	Fructose, mannose, galactose, starch, trehalose and sucrose metabolism	sucrose trehalose
	Glycolysis, gluconeogenesis, pyruvate metabolism	glycerate glucose-6-phosphate (G6P) glucose 3-phosphoglycerate phosphoenolpyruvate
	Nucleotide sugars, pentose metabolism	6-phosphogluconate lactobionate
Energy	Krebs cycle	citrate alpha ketoglutarate fumarate malate
	Oxidative phosphorylation	phosphoric acid
Lipid	Glycerolipid metabolism	glycerate O-phosphocolamine beta-glycerolphosphate glycerol 1-phosphate
Nucleotide	Purine metabolism, (hypo)xanthine/inosine containing	inosine
	Purine metabolism, adenine containing	adenosine adenosine 5'-monophosphate (AMP)
	Pyrimidine metabolism, cytidine containing	cytidine 5'-monophosphate (5'-CMP)
	Pyrimidine metabolism, uracil containing	uracil
added standards		myristic acid d27, D-glucose-C13 DL-isoleucine, DL-leucine-2,3,3-d



Appendix Figure 10.5: Multiple alignment of the PAH protein sequence of Homo sapiens (HsaPAH, accession number NP_000268.1), *D. melanogaster* (DmeHn, accession number NP_523963.2) and *An. gambiae* (AnogaPAH, accession number XP_315722.4) using the software praline (65% sequence identity).

Appendix Table 10.3. Survival of polyglutamine expressing *D. melanogaster* strains

gender	no. of PolyQs	n	mean (in days)	median (in days)	p- value (log-rank test)
male	httN25QEGFP_1a	22	68 ± 6	73 ± 4	0.02
	httN25QEGFP_1b	33	60 ± 6	73 ± 10	
	httN25QEGFP_2	52	66 ± 3	66 ± 2	
	httN97QEGFP_a	15	79 ± 5	82 ± 1	
	httN97QEGFP_b	40	45 ± 4	54 ± 7	
	httN97QEGFP_c	37	62 ± 4	66 ± 2	
female	httN25QEGFP_1a	32	82 ± 4	126 ± 1	< 0.001
	httN25QEGFP_1b	40	110 ± 4	108 ± 2	
	httN25QEGFP_2	77	94 ± 3	85 ± 3	
	httN97QEGFP_a	13	96 ± 3	101 ± 1	
	httN97QEGFP_b	43	72 ± 2	73 ± 3	
	httN97QEGFP_c	24	72 ± 4	73 ± 4	

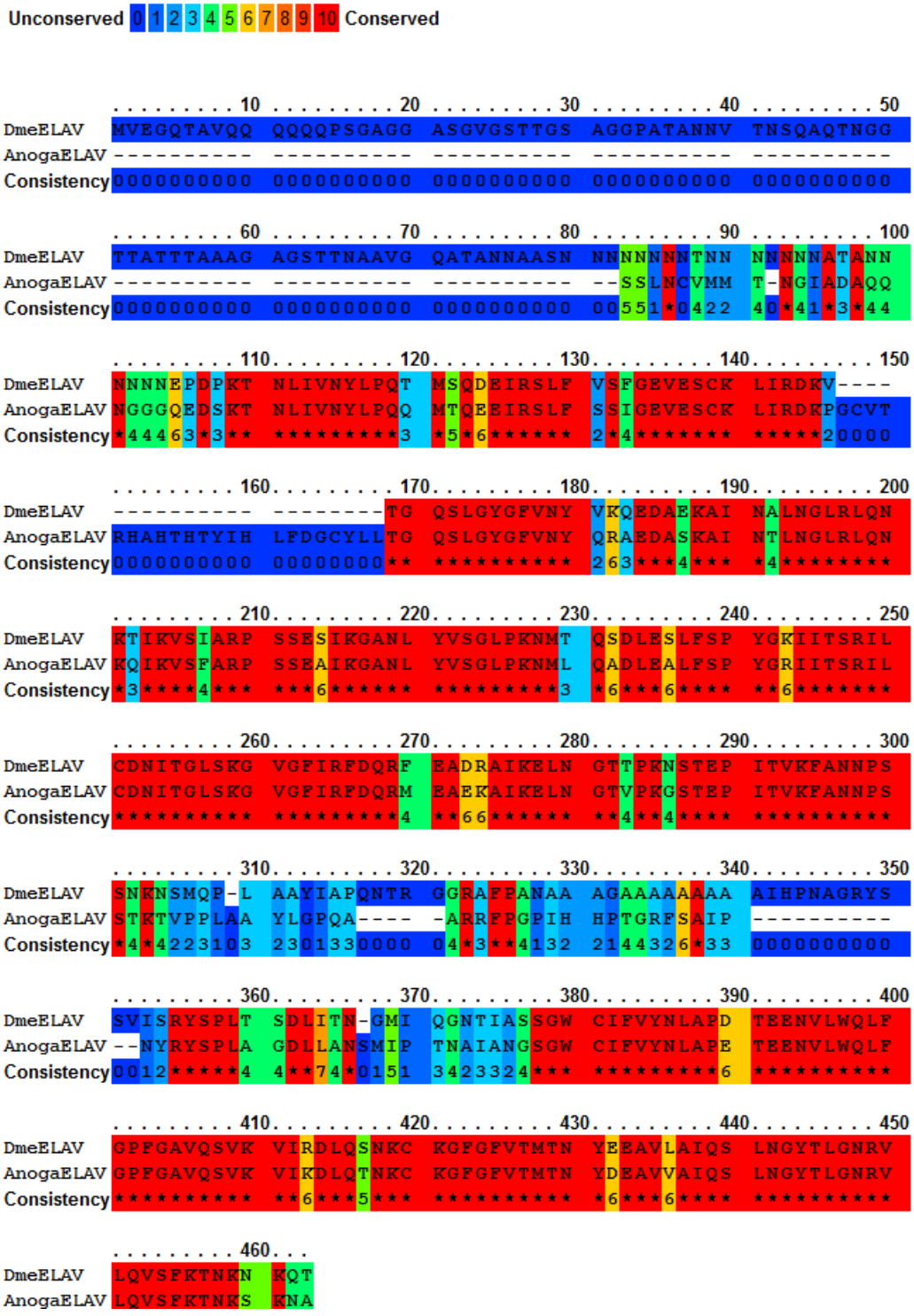
Appendix Table 10.4: Pairwise Comparisons of survival of polyglutamine expressing *D. melanogaster* males (log-rank test)

	97_a		97_b		97_c		25_1_a		25_1_b		25_2	
strain	χ^2	Sig.	χ^2	Sig.	χ^2	Sig.	χ^2	Sig.	χ^2	Sig.	χ^2	Sig.
97_a			32.79	.000	10.11	.001	1.13	.288	1.00	.317	7.09	.008
97_b	32.79	.000			12.04	.001	17.28	.000	13.77	.000	18.69	.000
97_c	10.11	.001	12.04	.001			2.78	.096	2.11	.147	.45	.505
25_1a	1.13	.288	17.28	.000	2.78	.096			.047	.829	1.65	.199
25_1b	1.00	.317	13.77	.000	2.11	.147	.05	.829			1.02	.313
25_2	7.09	.008	18.69	.000	.45	.505	1.65	.199	1.02	.313		

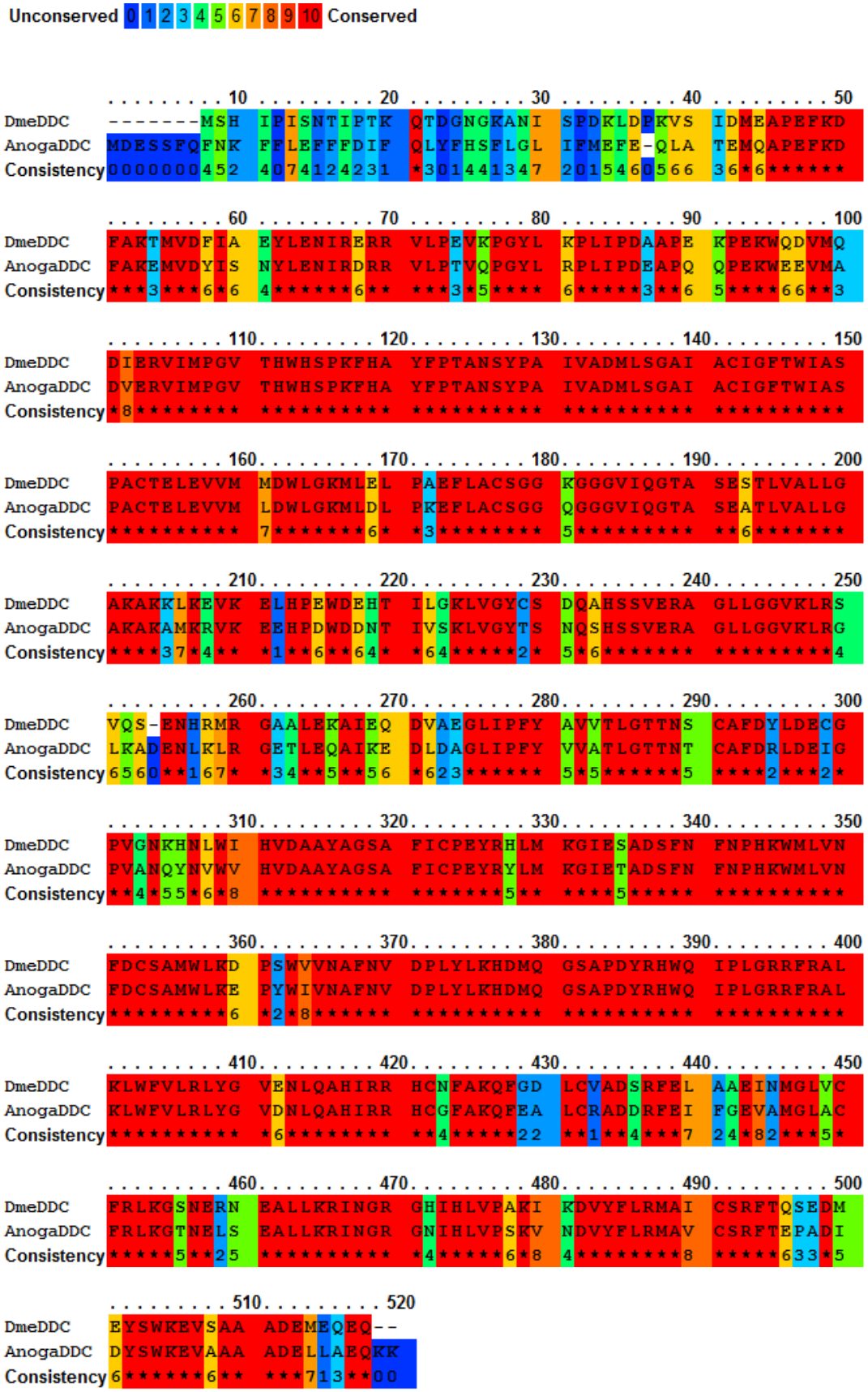
97a-c refers to strain httN97QEGFP a-c; 25_1a,b refers to strain httN25QEGFP_1_a,b; 25_2 refers to strain httN25QEGFP_2

Appendix Table 10.5: Pairwise Comparisons of survival of polyglutamine expressing *D. melanogaster* females (log-rank test)

	97_a		97_b		97_c		25_1_a		25_1_b		25_2	
strain	χ^2	Sig.	χ^2	Sig.	χ^2	Sig.	χ^2	Sig.	χ^2	Sig.	χ^2	Sig.
97_a			29.55	.000	22.76	.000	7.93	.005	19.10	.000	4.84	.028
97_b	29.55	.000			.85	.358	29.43	.000	64.00	.000	40.93	.000
97_c	22.76	.000	.85	.358			16.69	.000	49.28	.000	25.20	.000
25_1a	7.93	.005	29.43	.000	16.69	.000			41.97	.000	20.47	.000
25_1b	19.10	.000	64.00	.000	49.28	.000	41.97	.000			14.55	.000
25_2	4.84	.028	40.93	.000	25.20	.000	20.47	.000	14.55	.000		



Appendix Figure 10.6: Multiple alignment of the ELAV protein sequence of *D. melanogaster* (DmeELAV, accession number NP_001014462.1) and the predicted protein sequence of *An. gambiae* (AnogaELAV, accession number XP_319085.4) using the software praline (72% sequence identity).



Appendix Figure 10.7: Multiple alignment of the DDC protein sequence of *D. melanogaster* (DmeDDC, accession number NP_724163.1) and the predicted protein sequence of *An. gambiae* (AnogaDDC, accession number XP_319841.3) using the software praline (75% sequence identity).

Appendix Table 10.6: List of An. gambiae proteins which contain more than 14 continous polyglutamine stretches

protein	ontology	no. of polyQs	Molecular weight (kDa)
AGAP006758	Zink ion binding, nucleic acid bindin, intracellular	40 and 32	135.76
AGAP005244	unknown	33	82.39 (PA), 123 (PB)
AGAP005135	unknown	41	215
AGAP004734	DNA binding, nucleus	132	207.35
AGAP005881	Proteolysis, cystein-type peptidase activity	29	226.28
AGAP012385	Toll-like recptor	27	153.88
AGAP012422	Serine/threonine-protein kinase	22	45.30
AGAP005245	unknown	21	59.98
AGAP006800	Alcoholdehydrogenase transcription factor	19	60.95 (PA) and 68.23 (PB)
AGAP006454	Protein-binding	25	93.88
AGAP001755	unknown	14	15.18
AGAP006990	ARID/Bright DNA binding region	16	225.47
AGAP006376	bZIP transcription factor	19	51.33