TransMalariaBloc

Annex to the Final report

Project title: Malaria Transmission Blocking by Vaccines, Drugs and Immune Mosquitoes: Efficacy Assessment and Targets.

Grant agreement no: 223736

Reporting period: 01/12/2008 – 31/05/2013

Participant institutions: (1) Imperial College of Science, Technology and Medicine (Imperial); (2) University of Camerino (UNICAM); (3) Liverpool School of Tropical Medicine (LSTM); (4) Foundation for Research and Technology Hellas (FORTH); (5) Institut de Recherche pour le Développement (IRD), (6) Centre National de Recherche Scientifique et Technology – Institut de Recherche en Science de la Sante (IRSS); (7) University of Makerere (Makerere).

Participants: Maria-Gloria Basanez (Imperial), Oliver Billker (Imperial), George K. Christophides (Imperial), Fotis C. Kafatos (Imperial), Jacob Koella (Imperial), Robert E. Sinden (Imperial), Dina Vlachou (Imperial), Fulvio Esposito (UNICAM), Annette Habluetzel (UNICAM), Gareth Lycett (LSTM), Christos Louis (FORTH), Thanasis G. Loukeris (FORTH), Anna Cohuet (IRD/IRSS), Didier Fontenille (IRD), Jean-Bosco Ouedraogo (IRSS), Fred Kironde (Makerere), Jean- Baptist Kaddu (Makerere)

Project coordinator: George K. Christophides (Imperial)

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4.1 Final Publishable summary

a) Executive summary

TransMalariaBloc has made a significant contribution in the field of malaria elimination by establishing the foundations for the future exploitation of data derived by the project toward development of malaria transmission blocking interventions. Specifically, we have developed an analytical framework that can be used to determine the effective reservoir of infection in different endemic settings and the required efficacy of transmission blocking interventions. Using this framework and experimental data we have revealed that parasite density can strongly influence the reported success and efficacy of transmission-blocking interventions. A number of transmission blocking vaccine candidates have been tested, including the anti-Pfs25 that is currently at Phase 1 clinical trial and anti-P230 which can be an equally effective vaccine. We showed that Neem tree, among other, extracts have a great efficacy in blocking *P. falciparum* transmission and can complement the therapeutic properties of ACTs. We also revealed that specific HIV Protease Inhibitors cause strong *Plasmodium* developmental arrest, opening new avenues in the design of integrated HIV/AIDS and malaria chemotherapies. We demonstrated that antibiotics present in the human blood increase the mosquito capacity to transmit *P. falciparum*. Since antibiotic use in Africa is high and recommended in WHO-led disease elimination programmes, these findings are of particular importance to public health. Toward developing transgenic refractory mosquitoes, we generated lines overexpressing the anti-plasmodial mosquito complement pathway, which are now tested for their capacity to block or reduce parasite infection and hence affect malaria transmission. We also completed analysis of the genome-wide transcriptional responses of the *A. gambiae* and *A. arabiensis* to infections with *P. falciparum* populations sampled from infected children and identified an important role of GPCR signalling during infection. At the same time we identified several parasite genes showing strong phenotypes associated with mosquito midgut infection; some of them are currently explored as potential candidates for transmission blocking drugs and vaccines. Our work to characterise genotype*genotype interactions involved in *P. falciparum* infections of the *A. gambiae* midgut in natural settings has catalysed the development of novel technological platforms and yielded several candidates that are now being studied for their effect on parasite infections, including a group of proteins involved in maintaining the mosquito gut homeostasis. Finally we showed that *A. gambiae* and *P. falciparum* are highly resilient to seasonally variable, and occasionally extreme, weather conditions. In conclusion, TransMalariaBloc has precipitated existing and initiated new strong collaborations between

academic and research institutions in Europe and Africa, has educated and trained a number of graduates and research professionals in malaria research and has opened new research and exploitation avenues that could assist in the effort to eliminate malaria.

b) Summary of the project context and the main objectives

There are approximately 250 million malaria cases annually, causing ~1 million deaths of mainly children in Africa. In order to effectively control and eventually eradicate malaria, it is now recognized that blocking malaria transmission, which occurs through an obligatory passage of the malaria parasite through its mosquito vector, is of paramount importance. Indeed current effective control measures are largely based on reducing the vector populations or their contact with humans. However, these measures are thought to be inadequate as resistance to insecticides used for bed-net impregnation and indoor residual spraying is spreading fast, while mosquito vectors are thought to be evolving to change their biting habits, thus incapacitating indoor interventions. The TransMalariaBloc project was established to investigate novel means to stop malaria transmission by rendering mosquitoes unable to transmit the parasite. They include transmission-blocking vaccines and transmission-blocking drugs or remedies, which although administered to humans function to stop the parasite in the mosquito or to incapacitate the mosquito itself, and engineering mosquitoes to become resistant to parasite infections, by boosting their natural immune system or by supplying them with alternative resistance properties. The project aims at both a detailed biological understanding of the impact of such interventions on malaria transmission and epidemiology, and the development and testing of interventions.

c) Main S & T results and foreground

I. Summary of TransMalariaBloc S & T results and foreground

- We have developed malaria transmission and transmission-blocking models and showed that in high transmission areas current measures are inadequate and that transmission-blocking interventions can have a significant impact.
- We have established state-of-art infrastructure including experimental transmission facilities and transmission blocking assays in both the laboratory and the field. Especially the latter infrastructure is internationally unique.
- We have confirmed experimentally the efficacy of transmission blocking vaccines and showed that it is parasite density dependent. We have highlighted that anti P25 and P230 are the best transmission blocking vaccines to date.
- We have identified hundreds of transmission blocking compounds, including remedies and plant extracts. We have shown that extracts of the Neem tree are very potent transmission blockers and can complement current ACT therapies.
- We have identified the presence of antibiotics in the blood of malaria-infected children as a new risk of increasing disease transmission.
- We have identified several new transmission blocking targets in both parasites and mosquitoes, and characterized some of them in great detail. These targets should now enter a translational research path.
- We have generated genetically modified mosquitoes expressing immune factors that can eliminate parasites, the effect of which on infections remains to be confirmed.
- We have characterized the impact of infection, insecticides and environmental factors on malaria transmission, which will assist the design of transmission-blocking interventions.
- We have generated tools and contributed to e-infrastructures that can help the study of mosquito population biology and understanding the mosquito population structure.
- We have revealed a strong genetic component of the mosquito vectorial capacity that can help to identify the specific ecological and geographical characteristics of malaria transmission, and fine-tune the transmission blocking interventions.

II. WP S & T results and foreground

WP1: Manage TransMalariaBloc

WP #:	1	**Months:**						1-54
WP title:	Manage TransMalariaBloc							
Activity type:	RTD							
Participant #:	1	2	3	4	5	6	7	**Total**
Person months/participant:	8.53	0	0	0	0	0	0	**8.53**

Summary: TransMalariaBloc is a medium scale collaborative project that involves 7 beneficiaries from 6 countries, including 4 EU and 2 Disease Endemic Countries. Coordination and management is carried out by Dr George K. Christophides (Imperial College London). Within the first 18 months, the work was started as originally projected, necessary recruitments were timely made, the consortium and grant agreements were signed, and pre-financing was distributed to all beneficiaries. An external advisory board was established and their suggestions were implemented when appropriate. A kick-off and a subsequent annual meeting of the project, as well as a workshop of the entire FP7-funded malaria vector community were organised. The scope and achievements of TransMalariaBloc were presented by the coordinator in international meetings in seven different occasions, and published in a monthly magazine accessible to policy makers. A project website was created.

In the second reporting period, the coordinator visited 3 of the partner organizations and held several discussions with project participants, as part of his responsibility to coordinate and oversee the activities of the various WPs. He also reviewed formally the activities, milestones and deliverables of the project twice. These reviews formed the basis of a midterm report (which was discussed with the EVEC) and the current technical report. A successful coordination meeting was held at IRSS, Bobo Dioulasso. It included detailed scientific presentations of all the WPs and a full day field trip and engagement of all project participants with the activities at IRSS.

In the third reporting period, the coordinator visited 3 of the partner organizations (FORTH, LSTM and Makerere) and held discussions with project participants, as part of his responsibility to coordinate and oversee the activities of the various WPs. He also reviewed formally the activities, milestones and deliverables of the project twice, in preparation of the extension request and the current report. The former review led to adjustments and modifications, which were successfully implemented. A successful final reporting and future-planning meeting was held in Kolymbari, Crete. The meeting involved scientists from other institutions, which could play a role in a future schema. The accomplishments of TransMalariaBloc were presented to the scientific communities in various occasions.

Objectives of the reporting period: The first work package was set up to carry out coordination and management of the consortium and its activities. Specific objectives, all of which pertain in the current reporting period, include:

O1. Coordinate and oversee activities of various RTD WPs.

O2. Produce annual, mid-term and final reports for the EC.

O3. Promote networking activities of TransMalariaBloc investigators.

O4. Present overall achievements of TransMalariaBloc in international conferences.

Management and Administration (O1-O2): Before the start of the project the coordinator contacted all involved parties and ensured that appropriate structures are in place for timely recruitment of named as well as unnamed personnel and full deployment of TransMalariaBloc in December 2008. In the majority of cases, recruitments were done as initially anticipated; few deviations (due to recruitment complications) were within reasonable timeframes. WP leaders were also requested to submit an update of the research plan and Gantt chart that would take into account new data in the areas of research. Updates were received within December 08 and reviewed in the kick-off meeting in January 2009 (see below). A Consortium Agreement document

that included a detailed Data Exploitation Plan was drafted, circulated, revised and signed before the end November 08. Grant agreements were distributed to all beneficiaries in November 2009, signed until 22/12/2008 and submitted to EC. EC pre-financing budget was distributed to beneficiaries as soon as money was paid to Imperial College.

In coordination with other FP7-funded malaria vector projects and respective EC project officers a joint External Vector Experts Committee (EVEC) was established in September 2009 that included: C. Constantini (IRD/OCEAC, Yaounde), M. Coosemans (ITM, Antwerp), V. Do Rosario (University Of Lisboa, Lisbon), G. Gibson (Greenwich University, London), T. McLean (IVCC/LSTM, Liverpool), M. Jacobs-Lorena (Johns Hopkins, Baltimore), A. James (UCL, Irvine), S. James (FNIH/Grant Challenges, Bethesda), G. Putoto (Padua Teaching Hospital), N. Sagnon (CNRFP, Ouagadougou), Y. Toure (WHO/TDR, Geneva), S. Traore (MRTC, Bamako), and L. Zwiebel (Vanderbilt University, Nashville). EVEC and project officers met on 15-16/09/2009 following presentations of the five FP7-funded vector projects. The TransMalariaBloc project officer (A. Holtel) transmitted the EVEC comments/recommendations to the coordinator on 22/10/2010. These included: (a) “Concerns about the experimental design for the discovery of transmission-blocking drugs from crude extracts due to the number of partners and approaches, which all appear to be active in parallel. There is a strong need for organisational mechanisms in order to integrate these subprojects”; (b) “It has not been clear whether the ability to translate research into useful pharmacological entities has been adequately presented”; and (c) “Research agenda is too ambiguous, with too many objectives and thus clear and consensus driven prioritisation should be established.” The coordinator transmitted these comments to the concerned parties and when deemed appropriate corrective actions were taken in the following directions: (1) The activities of WP4 to identify transmission blocking drugs and remedies were further prioritised, and the number of extracts to be tested was reconsidered and reduced; (2) The number of genetically modified mosquito lines to be produced by WP5 was reduced; (4) The originally proposed establishment of *P. berghei* transmission cycle and related facilities in Burkina Faso were abandoned to avoid duplication of effort and building capacity that may not be necessary in the future. Instead emphasis on field P. falciparum research was strengthened and experiments related to *P. berghei* were conducted through visits of IRSS personnel to Imperial and UNICAM; (5) No immediate actions were considered necessary to identify mechanisms that would translate research into useful pharmacological entities; however, it was agreed that such mechanisms should be established in the near future and when additional funds become available. In that direction the coordinator has already made initial contacts with academic and private institutions, including the pharmaceutical companies GSK and Syngenta. Discussions also continued in the following months.

In September 2009 a modification was made related to the composition of the consortium. Dr L.C. Gouagna (IRD/IRSS) moved posts, which made it impossible to continue an effective collaboration with TransMalariaBloc. His responsibilities in the project were fully assumed by Dr A. Cohuet.

Man efforts throughout the project were closely monitored to ensure that resources are committed to the most appropriate areas, and that there are no major deviations from the original plan taking into account necessary updates due to the most recent developments in the field (**Table WP1.1**). A small deviation in actual compared to expected man-month effort is mainly due to minor delays in personnel recruitment and the fact that work by IRSS and Makerere on WP7 has not yet began. Man-effort reported by Makerere mainly concerned mosquito colonisation. It was anticipated that population biology work in Burkina Faso would start in early 2011, during the rainy season. Hence no corrective actions were deemed necessary. It must be noted that man-months reported in Table WP1.1 do not only involve personnel funded by the project; in some cases personnel funded by other sources contributed to the activities of the project, e.g. bioinformatics personnel at FORTH and Imperial involved with the development of a population genetics database and analysis tools.

During the second reporting period the coordinator has reviewed the activities, procedures and achievements of TransMalariaBloc in many occasions, through numerous email exchanges, telephone conversations and meetings with project investigators. He also visited 3 of the partner institutions including: LSTM (2/11/2010 and 25/02/2011) to discuss the activities of WP5 with G. Lycett, D. Amenya and A. Lynd; FORTH (5-6/04/2011) to discuss experimental aspects and future plans of WP4, WP5, WP6 and WP7 with T.G. Loukeris and C. Louis; and IRSS (5-9/11/2010) to

discuss field activities of WP3, AP4, WP7 and WP8 with J.B. Ouedraogo and A. Cohuet and to review infrastructure investments and gametocyte carrier recruitment procedures. In addition, the coordinator had informal meetings with T. Loukeris, K. Louis, and G. Lycett at the 2011 mosquito meeting in Kolymbari (Crete). He also had numerous meetings with Imperial investigators including R.E. Sinden, F.C. Kafatos, D. Vlachou, M.G. Basanez and O. Billker.

Twice during the current reporting period, the coordinator formally reviewed the accomplishments of each WP and the entire project: in June 2011, in preparation of the mid-term review and meeting with the EVEC; and in November/December 2011, in preparation of the 2nd report to the EC. Following the meeting with and formal feedback by the EVEC, the coordinator discussed in detail with the Makerere partners into identifying ways to speed up progress and enhance collaborative activities. Corrective actions were identified, including setting up an insectary facilities at the Makerere hospital and further training of personnel. He also discussed with investigators in WP5 about how to address issues with generation of transgenic mosquitoes suppressing Plasmodium development; a change of strategy was decided whereby expression of an inhibitor of metalloproteases rather serine proteases is pursued. Additional contribution of the FORTH partners to WP4 and WP6 has been also discussed and approved. After the November/December 2011 review, the coordinator in consultation with various partners have decided that a 6-12 month non-cost extension of the project shall be investigated, which would enable bringing some of the tools generated by WP3 and WP4 closest to further exploitation, provide necessary time for testing transgenic mosquitoes, and allow better integration of the Makerere partners. Following these discussions, the coordinator has preliminary discussed this possibility with the EC Project officer.

As part of his management responsibilities, the coordination oversees the man effort allocated to the various WPs by each of the partner institutions to ensure that effort and resources are allocated where most appropriate. The table below reports the effort in man-months that is directly incurred by the grant (**Table WP1.1**). Note that figures in Table WP1.1 are also reported in the heading of each WP.

Moreover, the coordinator with valuable support from WP leaders produced a mid-term review for the meeting with EVEC in June 2011 (**1D3**) and the present second report (**1D3**)

During the last reporting period the coordinator has reviewed the activities, milestones and products of TransMalariaBloc in several occasions. In addition, to numerous email exchanges, telephone and skype conferences he personally visited: IMBB/FORTH on 17-19/01/2012 to discuss with Dr Loukeris and Prof Louis about the progress of WP4 (HIV-PIs) and WP7 (Popbio and IDOMAL), respectively; the Sanger Institute on 28/03/2012 and 23/05/2013 to meet Dr Billker and discuss the progress on WP4 drug discovery; the LSTM on 30-31/05/2012 and 27/07/2012 to review the progress with the generation of transgenic mosquitoes (WP5) with Dr Lycett; the Makerere University on 01-05/04/2013 to examine completion of the insectary facilities and experimental transmission facilities at Iganga and sustainability of the programme. The visit to Uganda was particularly important and led to the consolidation of the malaria transmission facilities in Iganga.

Twice during the current reporting period, the coordinator formally reviewed the accomplishments of each WP and the entire project: in January/February 2012, in preparation of the extension request; and in September/October/November/December 2013, in preparation of the current, 3rd and final, report to the EC. In particular, following the review for the extension request, specific adjustments and modifications have been made which allowed opened new directions for the project and its future, including the inclusion of HIV-PIs and antibiotics in WP4, which are associated with a minor shift in fund allocation.

As part of his management responsibilities, the coordination oversees the man effort allocated to the various WPs by each of the partner institutions to ensure that effort and resources are allocated where most appropriate. The **Table WP1.1** below reports the effort in man-months that is directly incurred by the grant, while in **Table WP1.1** there is the total man-month effort that includes contribution from other sources including PhD, MRes and final year project students. Note that figures shown in Table WP1.3 are also reported in the heading of each WP.

Table WP1.1: Directly incurred man-month effort per partner and per WP									
WP	**Imperial**	**UNICAM**	**LSTM**	**FORTH**	**IRD**	**IRSS**	**MAKERERE**	**TOTAL**	**DEVIATION**
1	8.53	0	0	0	0	0	0	**8.53**	-3
2	56.3	0	12	6	0	0	0	**74.3**	4
3	56	0	0	0	41.49	54	79	**230.49**	45
4	31.29	107	0	35	1	112.7	0	**286.99**	69
5	10.8	0	41.5	53	0	0	0	**105.3**	-15
6	84	0	0	12	0	25	8	**129**	25
7	73.47	0	0	78	2	25	58.05	**236.52**	28
8	0	0	0	0	47	84	0	**131**	0
Total	320.39	107	53.5	184	91.49	300.7	145.05	**1202.13**	152

Networking activities and meetings (O3): A kick-off meeting was organised at Imperial College London soon after the start of the project on 16/01/2009. The meeting consisted of five sessions. Session 1 included presentations by Imperial College administrators (Ms Brooke Alasya and Ms Cecilie Hansen) on management, financial and technical reporting as well as legal obligations of beneficiaries (based on the consortium agreement), followed by questions and answers. In Session 2, partners in each WP met separately and discussed technical aspects of respective WPs; WP leaders who chaired these meetings then presented the outcomes of the discussions and the operational plan of each WP in Session 3. Session 4 included a meeting of the Executive Committee that discussed potential external reviewers who were then suggested to the EC Project officer for inclusion in the EVEC, while in Session 5 the coordinator summarised the outcomes of the day. Ms Megan Quinlan was invited to the meeting to give a 30 min presentation about MosqGuide, a WHO/TDR funded project aiming to develop and validate best practice guidance related to the range of requirements for deployment of genetically modified mosquitoes (www.mosqguide.org.uk). The meeting was characterised as highly successful and set out a very good pace for the start of project. Participants included: F.C. Kafatos, R.E. Sinden, J. Koella, M.-G. Basanez, D. Vlachou, G.K. Christophides, O. Billker, M. Lawniczak, R. MacCallum, T. Churcher, E. Dawes, and E. Bushell (Imperial); G. Lycett and A. Lynd (LSTM); A. Habluetzel (UNICAM); C. Louis and T.G. Loukeris (FORTH), A. Cohuet (IRD), J.-B. Ouedraogo (IRSS); and F. Kironde (Makerere).

On 15/09/2009, the coordinator (G.K. Christophides) and project officer (A. Holtel) of TransMalariaBloc together with the coordinator (A. Crisanti) and project officer (P. Tejedor Del Real) of InfraVec (FP7 funded infrastructure project) and the scientific officer of other FP7-funded vector projects (G. Quaglio) organised a Malaria Vector Workshop at Imperial College London. The objectives of this workshop were: (1) to introduce to EU and African vector researchers involved in five FP7-funded projects the widely accessible mosquito vector infrastructure of InfraVec; (2) to present and network all five FP7-funded malaria vector projects; (3) to establish a regular review process across the FP7-funded vector projects, conducted by a joint EVEC; and (4) to obtain strategic recommendations by EVEC for future EU funding in the area of vector research. In this workshop, the coordinator presented TransMalariaBloc and its objectives. TransMalariaBloc participants in this workshop included: R.E. Sinden, M.-G. Basanez, D. Vlachou, G.K. Christophides, M. Lawniczak, and T. Churcher (Imperial); G. Lycett (LSTM); C. Louis (FORTH), D. Fontenille (IRD) and A. Cohuet (IRD/IRSS); and J.-B. Ouedraogo (IRSS). In addition to meetings between TransMalariaBloc investigators, organised in the background of the workshop, detailed discussions and strong networking plans were established between the TransMalariaBloc coordinator and coordinators of three other FP7-funded projects: InfraVec, ANOPOAGE and ENAROMATIC, which soon led to a new application to the EC for additional funding that would further the activities of these projects, enable translational research as recommended by the EVEC and establish a pan-European vector network. The application has been unsuccessful.

A second TransMalariaBloc project meeting was organised in Heidelberg, Germany on 06/05/2010. Heidelberg was chosen as a very convenient place for most European partners and

also due to the BioMalPar (Biology of the Malaria Parasite) annual meeting organised by EviMalar, an FP7-funded European Network of Excellence, held in Heidelberg at the same time (03-05/05/2009). This gave the opportunity to participants to attend both meetings; most of TransMalariaBloc partners are also involved in the activities of EviMalar. The meeting agenda included a summary presentation by the coordinator of the overall activities and results of the project and more detailed presentations from WP package leaders, each followed by discussion. The meeting finished with closing remarks and summary by the coordinator. Participants included: R. Sinden, G.K. Christophides, M. Lawniczak and T. Churcher (Imperial); A. Habluetzel (UNICAM); C. Louis, T.G. Loukeris and E. Mitraka (FORTH); and A. Cohuet (IRD/IRSS). During that meeting it was decided that the next meeting would be held in Burkina Faso in late 2010 or early 2011.

During the first 18 months the coordinator has also conducted site visits to UNICAM (06-09/07/2009) and FORTH (27-28/07/2009), while additional site visits to European Institutions have been already conducted during the summer of 2010. The purpose of these visits was to meet with investigators in respective beneficiary institutions and discus/review the progress of the work. Reverse visits of partner investigators to meet with the coordinator at Imperial College also took place: T.G. Loukeris (FORTH; 16-22/10/2009) and C. Louis (FORTH; 08-09/02/2010. Additional networking activities between TransMalariaBloc researchers were promoted and facilitated by the coordinator and are reported in respective WPs.

An annual meeting of TransMalariaBloc was organized at IRSS (Bobo Dioulasso, Burkina Faso) on 6-8/11/2011 to promote networking between investigators/researchers, especially those relevant to field work since many of the deliverables of the project has reached the point of testing at field settings. The meeting was remarkably successful bringing together 13 project investigators and researchers including: G.K. Christophides (Imperial), O. Billker (Imperial), T. Churcher (Imperial), A Blagborough (Imperial), G. Lycett (LSTM), C. Louis (FORTH), E. Goulielmaki (FORTH), A. Habluetzel (UNICAM), A. Cohuet (IRD/IRSS), J.B. Ouedraogo (IRSS), S. Yerbana (UNICAM/IRSS), and D. Dari (IRSS). The 2.5-day meeting included: (1) discussion of reporting procedures between project members (day 1); (2) introduction about objectives and overall achievements of the entire project and of the IRSS partner by the coordinator and the director of IRSS (J.B. Ouedraogo), respectively. In addition to project participants, this session also involved the Secretary General and other officials of the Burkina Faso Ministry of Research and Innovation (day 2); (3) scientific presentations by all project participants (day 2); participation of all project participants in a field trip for identification of gametocyte carriers and detection of mosquito breeding sites (day 3); (4) critical discussion of the activities of the project and plans for continuation beyond the current grant (day 3).

From the start of the project, TransMalariaBloc partnered with a PhD Programme on Malaria and Human Development that is supported by “WHO Global Malaria Programme” and “TDR capacity strengthening” an coordinated by the project investigator Dr Annette Habluetzel (UNICAM). This partnership aimed to contribute to capacity strengthening in endemic countries and strengthen the activities of WP4 lead by UNICAM. To date, three PhD candidates developed part of their research within WP4, including Dr Serge Yerbanga, who graduated in June 2010 and is now employed at IRSS (Burkina Faso) working on activities of TransMalariaBloc, especially WP4. Three new PhD candidates were enrolled in 2011 and are involved in the second phase WP4 research at UNICAM.

Furthermore, an annual meeting of TransMalariaBloc was organized at end of the project to coincide with the international mosquito meeting in Kolymbari, Crete, on 16-19.07/2013. During the meeting the reporting, accomplishments and future of the project was discussed. The meeting was attended by G.K. Christophides (Imperial), D. Vlachou (Imperial), T. Habtewold (Imperial), J. Midega (Imperial), M. Gendrin (Imperial), M. Povelones (Imperial), T. Churcher (Imperial), H. Slater (Imperial), A Blagborough (Imperial), A. Lynd (LSTM), C. Louis (FORTH), T. G. Loukeris (FORTH), A. Cohuet (IRD) and L. Nabyonga (Makerere). Additional scientists were invited, who could play important roles in a future project, including L. Duchateau (University of Gent) and D. Yewhalaw (Jimma Univeristy). Prior to and in preparation for this annual meeting, another meeting took place in Paris on 17-18/05/2013 between G.K. Christophides, D. Vlachou and A. Cohuet, where the foundations of a continuation project were laid. Following this meeting G.K. Christophides discussed with S. Biswas (The Jenner Institute) specific ideas that emerged. In addition, the coordinator visited Ethiopia en route to Makerere on 27-31/03/2013 to discuss

putative collaborations in the future schema of TransMalariaBloc. This visit was very successful and led to the establishment of malaria transmission facilities in Gilde Gibe I dam, while a "mosquito village" is currently being build outside Jimma, where mosquito infection and behavioral experiments can be conducted.

Publications and public communication (O4): The coordinator presented the broad scope of TransMalariaBloc and communicated data that derived through the activities of the project to the scientific community by participating as an invited speaker in national and international workshops, meetings and conferences including: (1) Intl workshop on "Climate change and malaria", Imperial College London, 12-13/02/2009; (2) ESF Conference on "The impact of the environment on innate immunity: the threat of diseases", Obergurgl, Austria, 04-08/05/2009; (3) Intl workshop on malaria vector control, Camerino, Italy, 06-11/05/2009; (4) 4th Intl meeting on "Molecular and population biology of mosquitoes and other disease vectors", Kolymbari, Crete, Greece, 19-26/07/2009; (5) "Malaria vector workshop", London, UK, 15-16/09/2009; (6) Intl Conference of the Institute of Systems and Synthetics Biology of Imperial College London, London, UK, 11-12/11/2009; and (7) Spring meeting of the British Society of Parasitology, Cardiff, Wales, 30/03-01/04/2010.

The coordinator recently wrote an invited article about TransMalariaBloc in the research review issue of the EU parliamentary magazine to communicate the research to policy makers, society leaders and the broad public (http://www.theparliament.com/resrdigimag/issue13). This article summarizes the background and objectives of the project.

A website was created to communicate to the broader public the scope and achievements of the project (www.transmalariabloc.org). While main pages of the site are curated and maintained by the coordinator and his bioinformatics team, each WP leader is given access to curate and update pages that relate to each WP. In addition, each investigator and researchers can alone curate pages related to their profile. A news bulleting that reports latest developments in the field can be seen on the main page, whereas full stories can be accessed through the main menu. Protocols, procedures and a gallery of images related to the project have been added.

During the second reporting period, the coordinator presented all or some of the activities and achievements of the project in a total of 10 international conferences, seminars or focused meetings including: a departmental seminar at the University of Sheffield, UK (4/10/2010); a keynote lecture in a conference at Alexandropolis, Greece (18/10/2010); a focused cluster 3 meeting of the EVIMalR EC Network of Excellence in Stockholm, Sweden (26/10/10); a school seminar at LSTM, Liverpool (2/11/2010), a keynote lecture to tropical disease medical practitioners, nurses, medical policy makers and the public at the "Topics in Infection" meeting in London, UK (28/1/2011); a focused workshop on mosquito populations in Oxford, UK (30-31/3/2011); the InterMal ITN training course in Crete, Greece (4-5/4/2011); a institutional seminar at FORTH (6/4/2011); the annual meeting of the InfraVec EC Infrastructure project in London, UK (12-13/5/2011); and the joint meeting of all 6 current EC grant holders on malaria vectors organised together with the EVEC midterm review in Kolymbari, Crete (24/7/2011). In addition, during the second day of the annual project meeting in Bobo Diaoulasso, the coordinator and the IRSS director gave a press conference to national and local media and press about the activities of the project and the potential benefits to the Burkinabe research community and public.

During the third reporting period, the coordinator presented all or some of the activities and achievements of the project in international conferences, seminars or focused meetings including: SingMal meeting in Singapore (12-17/02/2012); BioMlaPar conference in Heidelberg (14-16/05/2012); Model Hosts conference in Rhodes (01-05/09/2012); EVIMalaR Cluster 3 meeting in Rome (02/12/2012); annual Royal Entomology Society meeting in Cambridge (31/01/2013); and Department of Genetics, University of Cambridge (08/03/2012). At the completion of the project, the coordinator had the opportunity to present twice to the most specializing audience the full breadth and accomplishments of the project including to the London Parasitology community (London Parasitology Club lecture series) in London School of Hygiene and Tropical Medicine (20/06/2013) and to the international mosquito community at the Mosquito Kolymbari meeting (19/07/2013).

WP2: Population and Evolutionary Biology of Transmission

WP #:	2	**Months:**						1-54
WP title:	Population and Evolutionary Biology of Transmission							
Activity type:	RTD							
Participant #:	1	2	3	4	5	6	7	**Total**
Person months/participant:	56.3	0	12	6	0	0	0	**74.3**

Summary: We have explored the effect of increasing parasite densities on mosquito mortality in the *Plasmodium berghei – Anopheles stephensi* transmission system. The estimated mortality parameters, together with those from our previous work on density dependence during sporogony have been used to develop and calibrate a stochastic mathematical model. We have collated and analysed published (aggregate) data on the relationships between densities of consecutive parasite stages during sporogony in other experimental systems and natural parasite–vector combinations. The end result is an analytical framework that can be used to determine the effective reservoir of infection in different endemic settings and the required efficacy of interventions in order to block human–mosquito transmission in regions where malaria is transmitted by *Anopheles gambiae*. The impact of the mosquito-parasite interactions on these models has been investigated, and the framework has been developed to extend those to incorporate the specific mosquito and parasite genotypes. Finally, the evolutionary impact of these interactions on the epidemiology of malaria has been studied by considering the specific ecological characteristics of disease transmission settings.

Objectives

O1. Identify and quantify the strength of the main density-dependent mechanisms (and which life-stage they act upon) that regulate parasite population abundance within the mosquito in experimental and natural systems that can be exploited by transmission-blocking interventions to effect measurable reductions in transmission to and from vectors;

O2. Evaluate the amount and distribution of variability in density dependence that is present in experimental and natural combinations by analysing individual mosquito data wherever possible;

O3. Quantify, for the main *Plasmodium-Anopheles* combinations investigated in O2, the effort required in decreasing the density of particular life-stages;

O4. Measure the variability among parasite isolates in their ability to infect mosquitoes and in their (density-dependent) transition probabilities up to the oocyst/sporozoite stage, and evaluate the difference in infectivity of parasite isolates on several mosquito genotypes and species; to effect a given decrease in transmission, ranging from modest reductions to complete blocking;

O5. Incorporate the processes identified into full transmission models encompassing other aspects of vector biology (e.g., vector/host ratio; per mosquito biting rate on humans; anthropophagy; female survival) in order to evaluate interactions between the variables that determine transmission.

Modification: The development of a database to handle mosquito population genetic data is now handled by and reported in WP7 for consistency. The original deliverables **2D2** of WP2 and **7D4** of WP7, which refer to the same database resource, are now merged into one deliverable referred to as **2D2&7D4**.

O1, O2. A literature review provided data to explore the nonlinear phenomena we had found in the *P. berghei–An. stephensi* system in other parasite–vector combinations. Data were obtained from other model systems, both rodent (*P. chabaudi*, *P. yoelli*, and *P. vinckei* in *An. stephensi*) and avian (*P. gallinaceum* in *Aedes aegypti*), as well as from *Plasmodium* species which infect humans (*P. vivax* in *An. dirus*, *An. minimus* and *An. sawadwongporni*; *P. falciparum in An. gambiae*). The data comprised parasite densities on specific days post-blood feed, allowing the exploration of specific life-stage transitions. Published datasets were somewhat limited in that they often consisted of parasite density data for only one or two life-stages. The majority of the data were available only in aggregate (mean density, prevalence) format. Nested models (linear, hyperbolic –

saturating– and sigmoid –S-shape–) were fitted to the data by maximum likelihood and the most parsimonious yet adequate models were selected. The magnitude of the parameters and their associated uncertainties were estimated. The obtained results **(Task 1)** demonstrate that although there is evidence of density dependence during sporogony in other parasite–vector combinations, the relationship between life-stages can be species, study, and life-stage specific. A database that host population data has been constructed (see WP7).

O3. Data from mosquito feeding experiments conducted on naturally found parasite-vector combinations from across Africa were collated to generate a dataset of more than 18,000 mosquitoes which had been fed on blood from more than 400 different human patients. Gametocytaemia had been estimated by either microscopy or quantitative nucleic acid sequence-based amplification. Mosquito infectivity had been assessed by both the presence of viable oocysts and the number of oocysts identified in infected mosquitoes. Novel Bayesian methods to estimate gametocytaemia measurement error and its influence on the shape and magnitude of the nonlinear processes previously described have been developed. The relationship between gametocytaemia and oocyst presence and density was best described by a sigmoidal curve, indicating that sporogonic development is restricted at both low and high gametocyte densities. The results have implications for transmission-blocking interventions (TBIs) and for prospects of malaria elimination **(Task 2).** The processes regulating the development of the malaria parasite within the mosquito may influence the success of TBIs. An individual-based stochastic mathematical model was used to investigate the combined impact of these multiple regulatory processes and examine how TBIs which target different parasite life-stages within the mosquito may influence overall parasite transmission. This work has been published in *Malaria Journal* in 2010 (Churcher et al., 2010) (**2D4**).

O4. The outcomes of sympatric and allopatric parasite-vector combinations were analysed in collaboration with Dr Anna Cohuet (WP7) and a paper was published in *PLoS One* (**2D6**) (Harris et al., 2012).

O5. The processes regulating the development of the malaria parasite within the mosquito may influence the success of TBIs. An individual-based stochastic mathematical model was used to investigate the combined impact of these multiple regulatory processes and examine how TBIs which target different parasite life-stages within the mosquito may influence overall parasite transmission. Under certain scenarios, a partially effective TBI could enhance transmission by increasing the number of infectious bites made by a mosquito during its lifetime whilst failing to sufficiently reduce its infectivity. However, interventions that reduce ookinete density beneath a threshold level are likely to have auxiliary benefits, as transmission would be further reduced by density-dependent processes that restrict sporogonic development at low parasite densities. The best parasite molecular targets are likely to vary between different epidemiological settings (**Tasks 4, 6**). In collaboration with other groups at Imperial, an individual-based simulation model has been developed for *P. falciparum* transmission incorporating the major vector species in Africa (*An. gambiae s.s., An. arabiensis and An. funestus*). Parameters were obtained by fitting to parasite prevalence data from 34 transmission settings across Africa. Simulated interventions to date include the switch to artemisinin-combination therapy (ACT), increased coverage of long-lasting insecticide treated nets (LLIN), additional rounds of indoor residual spraying (IRS), mass screening and treatment (MSAT), and a future RTS,S/AS01 vaccine and a partially effective transmission blocking vaccine in six settings with varying transmission intensity (EIR varying from 3 to 675 infectious bites per person per year). This work has been published in *PLoS Medicine* in 2010 (Griffin et al., 2010). A further manuscript developing the population dynamics of the mosquito (from eggs to adults) for integration into full-transmission cycle models has been published in *Parasites & Vectors* (White et al., 2011). A paper on spatial targeting of hot spots of malaria transmission for optimal allocation of interventions for control and elimination has been published in *PLoS Medicine* (Bousema et al., 2012). These publications together contribute to deliverable **2D5**.

We developed statistical models describing the relationship between gametocytes and oocysts from data of field studies in natural transmission settings. Transmission reduction is a key component of global efforts to control and eliminate malaria; yet, it is unclear how the density of transmission stages (gametocytes) influences the resulting proportion of mosquitoes infected. We

assessed human (gametocyte carriers) to mosquito transmission (oocyst stage) using 171 direct mosquito feeding assays conducted in study sites in Burkina Faso and Kenya. We found that *Plasmodium falciparum* infects *Anopheles gambiae* very efficiently at low densities (4% of mosquitoes are infected when they ingest blood with as few as 1 gametocyte per µl of blood), although more than 200 gametocytes/µl are required to increase infection further (**Figure 2.1**). We also confirmed that, as suggested by our laboratory-derived data, the relationship between these two variables is strongly non-linear. In the study site in Burkina Faso, we found that children harbour more gametocytes than adults, but this does not necessarily translates into a greater contribution to transmission than that of adults because of the non-linear relationship between gametocyte density and mosquito infection. Our analytical framework can be used to determine the effective reservoir of infection in different endemic settings. Interventions reducing gametocyte density need to be highly effective in order to halt human–mosquito transmission (**Deliverable 11 (2D8)**). Their use can be optimised by targeting those contributing most to transmission (Churcher et al., 2013).

Milestone 30 (2M5) and deliverable 9 (2D6). Based on information reported in (Harris et al., 2012), we have started developing evolutionary models of mosquito-parasite interactions (Slater et al., in preparation). Although mathematical modelling has been extensively applied to the investigation of the epidemiological consequences of malaria control, less attention has been paid to the modelling of vector–parasite interaction dynamics and the effect on these dynamics of antimalarial measures. We have reviewed the evidence on how malaria infection and transmission relate to vector biting rate, vector mortality and vector fecundity, and the potential mechanisms governing the changes in the life-history traits of both species. The ultimate aim is to modify classic malaria models to investigate the potential epidemiological implications of such interactions. These models will be extended for the study of genotype–genotype interactions.

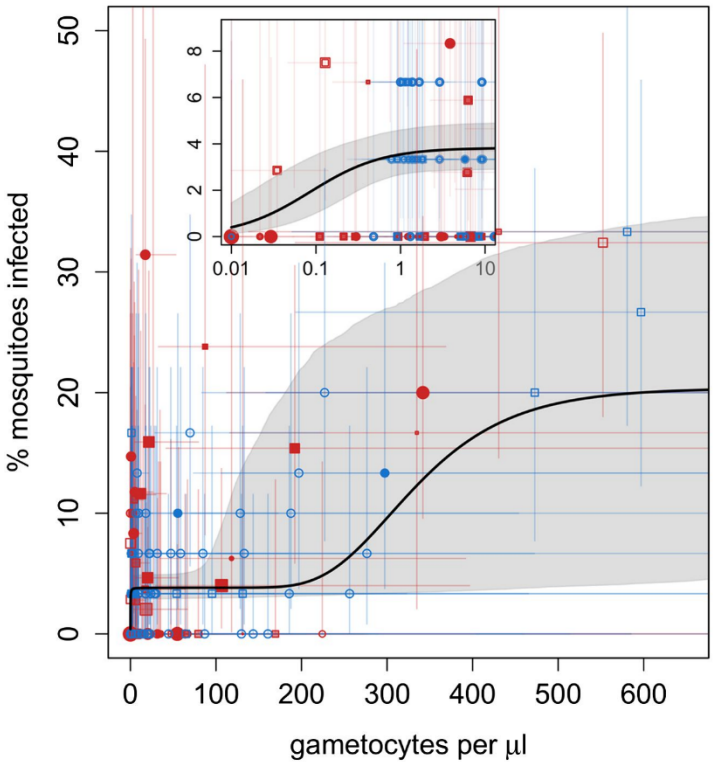

Figure 2.1 – The relationship between *Plasmodium falciparum* gametocyte density and *Anopheles gambiae* infection. Point colour, shading, and shape denote characteristics of the blood donor, such as location (blue = Burkina Faso; red = Kenya), asexual parasite density as measured by microscopy (no fill colour = none detectable, light shading = 1–1000 parasites per microlitre, dark shading ≥1000 µl−1), or host age (<6 years old = square, ≥6 years old = circle). The size of the point is proportional to the number of mosquitoes dissected. Coloured horizontal and vertical lines indicate 95% Bayesian credible intervals (CIs) around point estimates. The solid black line indicates the best-fit model, whereas the grey shaded area indicates the uncertainty around this line. The inset shows the relationship at very low gametocyte densities (on a logarithmic scale). The outputs show the shape of the relationship for a child with no detectable asexual parasites.

Milestone 31 (2M6) and Deliverable 10 (2D7). Evolutionary model(s) of malaria epidemiology and control (Pollitt et al., 2013): The usefulness of using evolutionary and ecological frameworks to understand the dynamics of infectious diseases is gaining increasing recognition. Integrating evolutionary ecology and infectious disease epidemiology is challenging because within-host

dynamics can have counterintuitive consequences for between-host transmission, especially for vector-borne parasites. A major obstacle to linking within- and between-host processes is that the drivers of the relationships between the density, virulence, and fitness of parasites are poorly understood. By experimentally manipulating the intensity of rodent malaria (*P. berghei*) infections in *A. stephensi* under different environmental conditions, we showed that parasites experience substantial density-dependent fitness costs because crowding reduces both parasite proliferation and vector survival. We use our data to predict how interactions between parasite density and vector environmental conditions shape within-vector processes and onward transmission. Our model predicts that density-dependent processes can have substantial and unexpected effects on the transmission potential of vector-borne disease, which should be considered in the development and evaluation of transmission-blocking interventions. We are currently performing final infection experiments of GM mosquitoes generated by WP5. Data from these experiments were integrated into these evolutionary models to analyse the impact of the GM intervention on epidemiology of malaria.

Finally, we have developed statistical models for investigating the temporal and micro-spatial heterogeneity in the distribution of *Anopheles* vectors of malaria along the Kenyan coast (Walker et al., 2013). Data on *A. gambiae* and *A. funestus* collected from households in Kilifi, Kenya, were analysed using polynomial distributed lag generalized linear mixed models (PDL GLMMs). Anopheline density was positively and significantly associated with amount of rainfall between 4 to 47 days, negatively and significantly associated with maximum daily temperature between 5 and 35 days, and positively and significantly associated with maximum daily temperature between 29 and 48 days in the past (depending on *Anopheles* species). Multiple-occupancy households harboured greater mosquito numbers than single-occupancy households. A significant degree of mosquito clustering within households was identified. The PDL GLMMs developed represent a generalizable framework for facilitating detailed understanding of the determinants of the spatio-temporal distribution of *Anopheles* vectors. Such understanding will facilitate delivery of targeted and cost-effective anti-vectorial interventions against malaria

WP3: Transmission Blocking Vaccines (TBV)

WP #:	3	**Months:**						1-54
WP title:	Transmission Blocking Vaccines (TBV)							
Activity type:	RTD							
Participant #:	1	2	3	4	5	6	7	**Total**
Person months/participant:	56	0	0	0	41.49	54	79	**230.49**

Summary: WP3 aimed to investigate whether, how, and to what extent, different infection intensities and genetic variability of parasite and vector input upon TBV efficacy and to provide search for candidates for the future development of potent TBVs. At the close of this reporting period, all objectives have been achieved. Significant infrastructure development at both Burkina Faso and Uganda has been performed to enhance field-based malaria transmission research. A large number of promising young scientists have been trained at both sites, with the eventual aim of translating interdisciplinary research into the realities of implementing control in Africa. In the close of the project, the site in Burkina Faso continues to be unarguably one of the best currently available to the community to study malarial transmission, and will be self-sustainable for the near future, while our new site in Iganga, Uganda is set to become a great place to perform transmission blocking vaccine trials. Experiments investigating the effect of gametocyte density ability to elicit transmission-blockade were performed using *P. berghei* and *P. falciparum* and showed that parasite density can strongly influence the reported success and efficacy of transmission-blocking interventions. This has significant implications on the accurate and comparable assessment of existing and novel TBIs. To examine *P. falciparum* polymorphism, we studied multiplicity of infection based on polymorphism of isolates from children afflicted with mild/complicated malaria. To examine *An. gambiae* polymorphisms, we additionally conducted field studies of *Anopheles* in Uganda. We continued studies in the laboratory and the field to identify

new TBV candidates. In *P. berghei*, we identified numerous new potential sexual-stage antigens. We also produced immune sera to examine these potential antigens further to produce new TBVs. Parallel studies testing serum in *P.falciparum* strongly induced TB efficacy. Building on the promising results, further studies to identify new TBV candidates (Pfs25, P320, Pvs25) were conducted, with successful results observed and currently awaiting publication. A wider range of novel TBV targets and antigens (identified proteomic datasets and bioinformatics analysis) have been identified, with ongoing experiments to confirm their efficacy. In Burkina Faso, serum resulting from a Phase 1 clinical trial in US for Pfs25 was tested for its efficacy against P. falciparum field isolates. This was compared to genetically diverse parasites on experiments carried out in Thailand. The genetic diversity of the parasites was related to efficacy observed. This first study on efficacy of human serum immunized for a TBV candidate on field parasite isolates was recently published.

Objectives:

O1. Capacity building at Burkina Faso and Uganda through infrastructure development, and onsite/overseas training.

O2. Generation of laboratory data which together with existing data would allow the development of models examining whether parasite density modulates the impact of TB antibodies in *P. berghei* /*P. falciparum.*

O3. Genotyping parasites and mosquitoes to establish the diversity of vector and parasite populations at different locations.

O4. Assess the impact of TB antibodies on parasite development with variant populations of parasite/vector.

O5. Discover and characterize new targets for potential vaccine candidates, which would extend TBV weaponry and allow further validation of models (link with WP6).

Modifications: Continued from the previous reporting period, enhancements to O5 have continued in this reporting period.

O1 (3D1, 3M1):

Within the first reporting period of the WP, to facilitate further TBV analysis in active centres of field-based research, we have successfully completed the instalment of a mosquito rearing facility in Makerere, Uganda. Infrastructure and training has also been successfully improved in Burkina Faso, where a full team was trained for transmission-blocking activities and staff members were trained by a visiting Imperial researcher in colonizing and rearing *Anopheles* mosquitoes, egg handling and storage, and membrane feeding. In addition, a PhD student from Bobo Dioulasso has been trained for 6 months at Imperial College, London and presentations were given by members of IRRS to the 2011 coordination meeting of TransMalariaBloc (7-8/11/2012). Furthermore, in order to facilitate the analysis of TBV interventions within this WP fully, as well as additional TB-related work proposed in other WPs, it had been necessary to use modest investment to improve technical support and on-site facilities in both Burkina Faso and Uganda. To this end the instalment of a mosquito rearing and membrane feeding facility in Makerere has been completed. We also set up a fine building facility (45 m^2) for mosquito rearing and infections at Iganga, Uganda, next to our existing malaria workstation within the premises of the Iganga Hospital. These facilities now offer the possibility for state of the art research in transmission blocking interventions, as they combine mosquito insectary, molecular biology laboratory, clinic, vaccination rooms, patient rooms equipped with beds, offices and well trained personnel. The combination of our facilities in Uganda and Burkina Faso form a unique infrastructure that can be used in research towards studying malaria transmission and developing interventions for transmission blocking.

O2 (3D2, 3D4, 3M2, 3M3): Initially, a student started his PhD on the project and was trained for all the steps of the protocol in Bobo Dioulasso. He also benefited a training of 6 months (5 months during the reporting period) at Imperial College, London for experiments using the murine parasite *P. berghei* and a 2 weeks training in Cameroon for isolation of individual oocysts and genotyping. The student then transferred the technology of individual oocyst isolation and genotyping to one technician in the IRSS lab. In addition, experiments to investigate density dependence of parasites

on transmission-blockade using *P. berghei* to identify the effect of gametocyte density and antibody concentration on ability to elicit transmission-blockade were accomplished. Complimentary experiments using *P. falciparum* and the anti-Pfs25 mAb 4B7 were also performed and presented at the 2011 meeting of TransMalariaBloc, and a manuscript is in preparation. Then, data was passed to WP2. Additional studies to assess the density dependence of Plasmodium transmission, both in vivo and in vitro have been also performed. Specifically, complementary experiments in the *P. falciparum-A. gambiae*, and the model rodent malaria parasite *P. berghei-A. stephensi* systems have been performed, using both the anti-Pfs25 mAb 4B7 and the anti-Pbs28 mAb 13.1. Results demonstrate that parasite density can strongly influence the reported success and efficacy of transmission-blocking interventions (TBIs) in the SMFA (Blagborough et al., 2013; Churcher et al., 2012). Importantly, results also indicate that the amount and method of sample dilution (especially in the P. berghei model) can have a highly significant impact on sporogonic development and the reported result of SMFAs, and therefore, the reported potency of TBIs. This has significant implications on the accurate and comparable assessment of existing and novel TBIs. A manuscript describing these results is currently in preparation.

O5 (3D6, 3M7): In Uganda, in order to identify *P. falciparum* populations that can be used in TBV experiments as well as experiments described in other work packages (WP6 and WP7), surveillance work has been carried out at Makerere demographic study site (Iganga, 100 km est of Kampala). We also performed studies to identify new TBV candidates. We immunized mice anti-PbHAP2 with two other known TB antigens (Pbs28/AgAPN1) to assess transmission blockade in a synergistic fashion. Immunisations are completed within mice, and quantification of TB is underway. Parallel studies at IRSS examined the impact of single and combinations of antibodies induced by equivalent *P. falciparum* antigens. Of 5 antigens tested in *P. falciparum*, 2 induced a strong response. Efficacy reached 100% from 62.5 to 250 µg/ml Ab concentration depending on intensity. We are currently modelling efficacy versus intensity and antibody concentration. To further identify novel targets, we identified potential new targets from proteomic datasets and bioinformatic analysis (Wass et al., 2012). We generated numerous insect-expression constructs to induce immune responses to these antigens in laboratory animals. We have additionally produced protein derived from 4 potential antigens in *E. coli* expression systems. Additional, high throughput, laboratory and field studies for identification of new targets of TBVs, TBDs, TBRs and TBMs are reported in WP6.

To improve methods of vaccine generation and delivery for the induction of antibodies with transmission-blocking activity, we successfully tested a Baculovirus Dual Expression System that functions as both a subunit and DNA based vaccine and can be delivered via both intranasal and intramuscular immunization (Blagborough et al., 2010) and immunization with an adenovirus-MVA vaccine platform as an alternative approach to subunit vaccines

Building on the promising results described in the previous reporting period, studies to identify new TBV candidates (and novel potent ways to deliver TBVs) continued. Previously, mice were immunized with multiple constructs and SMFAs were performed examining the efficacy of single TBVs in comparison with potency observed when multiple TBVs were used in combination. The TBV candidates examined were PbHAP2, Pbs28 and PgAPN1. Only additive TB effects were observed, with no evidence of synergy when combining anti-gamete and anti-ookinete TBIs. Parallel studies examining the efficacy of 5 main TBV candidates, tested by mice immunization and addition of purified antibodies to *P; falciparum* gametocyte containing blood, revealed high levels of efficacy for Pfs25 and Pfs230 whereas other candidates ($Pfs48/45_{+NGln}$ and $Pfs48/45_{-NGln}$, APN1) revealed low efficacy. A manuscript reporting this first parallel comparison of the main TBV candidates using similar delivery systems against lab and field isolates is submitted to the journal "Science Translational Medicine". Efficacy of mixes of antibodies, as well as antibodies from double immunizations are currently under further investigation.

Numerous studies have been performed at IC to identify potential novel TBV targets and antigens identified from proteomic datasets and bioinformatics analysis. We have currently identified 42 novel, uninvestigated, potential TBV targets on the surface of the male gamete. These candidates are undergoing validation for further TB studies. In addition, three insect-expression based constructs to induce immune responses in mice have been constructed, and are currently under investigation. Multiple TBV targets have also been expressed in recombinant form in *E. coli* to

generate anti-serum. Future studies will involve assessing their potency in direct feeds (DFA) and the SMFA.

Additional studies have been performed against the *P. vivax* TBV target Pvs25. Studies comparing the potency of recombinant Pvs25 with different adjuvants (e.g. Abisco/Alhydrogel) and adenoviral delivery platforms have successfully been performed (manuscript in preparation). Experiments using the Baculovirus Dual Expression system (Blagborough et al., 2010), examining the utility of combining an anti-Pvs25 TBV with a pre-erythrocytic anti-malarial vaccine have also been successfully completed, indicating potent TB activity (manuscript in preparation).

O3-4 (3D3, 3D5, 3M4, 3M5). In Burkina Faso, the serum resulting from a phase 1 clinical trial in US for Pfs25 was tested for its efficacy against *P. falciparum* field isolates. This was compared to parallel experiments carried out in Thailand. The genetic diversity of the parasites was tested by microsatellites on blood stages parasites. Burkina samples revealed a large number of haplotypes (2 to 9), whereas Thai samples showed infections with 1 to 2 haplotypes. In spite of this major difference in parasite diversity in Burkina and Thai infected blood samples, similar efficacy of the immunized serum was demonstrated. Between 34 to 97% fewer oocysts were observed, however the efficacy at reducing oocyst prevalence was lower (0 to 82 %). The limited reduction of prevalence, compared to reduction of intensity in some assays might be due to high levels of infections generated by selection of the highest gametocytes densities. This first study on efficacy of human serum immunized for a TBV candidate on field parasite isolates was recently published (Da et al., 2013).

We also studied *P. falciparum* polymorphisms and multiplicity of infection (MOI) in parasite isolates from children afflicted with mild and complicated malaria at Mulago Hospital in Kampala (Kiwuwa et al., 2013). Two Makerere lecturers (Dr F. Namusoke and Dr S. Kiwuwa) trained in DNA genotyping technology and undertaking PhD studies in malaria molecular biology during the past few years have completed their PhD studies during this period.

We conducted field studies of *Anopheles* mosquitoes in Iganga and Bugiri district of eastern Uganda and have advanced well in ecological and insecticide resistance studies of this vector in this region. The districts of Iganga and Bugiri are neighbouring to each other. Iganga is where our team has carried out a blood-stage phase -2 malaria vaccine (GMZ2) trial among 250 children aged 1 to 6 years. The DNA of about 500 mosquitoes from Iganga and Bugiri has been analyzed. In the DNA encoding para-type sodium channel protein, we find mutation (East-African mutation, L1040S) frequency of 43 and 8 % in *A. gambiae* and *A. arabiensis*, respectively. For *An arabiensis* (Bugiri rice fields), knock down resistance to lambdacyhalothrin and DDT are respectively 10 and 25 %. Further, we collaborated with the Ugandan Ministry of Health to carry out genotyping of DNA from *Anopheles* mosquitoes (n=500) collected in four different regions of Uganda under a comprehensive study of efficacy of different ITNs (bednet) brands (Okia et al., 2013). In addition to junior technicians and two master's degree trainees (Dr B. Straton and Ms M. Musoke) who have trained in *Anopheles* mosquito work, a postdoctoral fellow Dr Ndagire returned to Uganda and joined in this research by initially undertaking a two month exchange training visit to the lab of Prof Christophides (Imperial). Dr Ndagire now continues her research under internal scholarship awards from Makerere College of Health Sciences (MakCHS-CORE grant of NIH, USA and MakCHS-Wellcome Trust support, THRIVE).

WP4: Transmission Blocking Drugs & Remedies (D&R)

WP #:	4	**Months:**						1-54
WP title:	Transmission Blocking Drugs & Remedies (D&R)							
Activity type:	RTD							
Participant #:	1	2	3	4	5	6	7	**Total**
Person months/participant:	31.29	107	0	35	1	112.7	0	**286.99**

Summary: WP4 was set to address the question whether it is feasible to design combination drugs

with an increased TB activity in respect to currently used ACT's, by adding components that act on the parasite stages developing in the vector after assumption of an infectious/treated bloodmeal or by designing plant extract combinations with characterized anti-plasmodial multi-stage TB activity. During the first reporting period, screening of 72 plant extracts and 32 known schizonticidal compounds revealed TB activity of 36 extracts/ compounds. Six extracts demonstrated *in vivo* TB activity in the murine model. We showed that combining plant extracts we previously found to affect early stages of Plasmodium development in mosquitoes could have additive but no synergistic impacts on malaria transmission blocking. We then tested the effects in field conditions of combining our most TB active extract, NeemAzal®, which derives from the Neem tree, with ACT. Our results showed that NeemAzal could indeed complement the therapeutic properties of ACTs in P. falciparum by largely blocking transmission to mosquito. Molecular characterisation of the active fractions of NeemAzal® revealed that, besides azadirachtin A, additional azadirachtins and limonoids are very important in the documented TB activity of this extract. Finally, we extended WP4 to test the TB potential of HIV Protease Inhibitors (HIV-PIs) and to examine the potential impact of antibiotic treatment on malaria transmission. Our data revealed that treatment with three HIV-PIs induces strong Plasmodium developmental arrest at the early mosquito stages, opening new avenues in the design of integrated HIV/AIDS and malaria chemotherapies. We also demonstrated that antibiotics present at therapeutic concentrations in human blood increase the mosquito's capacity to transmit *P. falciparum*. Since antibiotic use in Africa is high and recommended in WHO-led disease elimination programmes, these findings are of particular importance to public health.

Objectives: O1. To select most active transmission blocking (TB) fractions from a panel of antiplasmodial terpenoid rich extracts/remedies of plant origin and to assess TB potential of antiplasmodial enzyme inhibitors acting on known targets in parasite transmission stages and to select terpenoid rich fractions with prominent insecticidal / bioregulatory activity against the mosquito vector.

O2. To characterise selected TB drugs/compounds/remedies (D&R) by target stage and by affected cell component.

O3. To estimate performance under field conditions, by assessing (in laboratory studies) the influence of i) D&R (including currently used ACT) dosage, ii) gametocytemia, iii) repeated blood meals, iv) mosquitotoxic effects, on TB efficacy of D&R.

O4. To evaluate the additional benefits of combining currently used TB drugs (including ACT) and D&R emerging from the WP4.

O5. To evaluate the additional benefits of combining currently used TB drugs (including ACT) and D&R (O1 to O5) on *Plasmodium falciparum* field isolates / colonies of field *Anopheles* spp.

Modifications:

O1-3 were dealt with extensively and reported in the previous periodic reports. In the 3rd period we report the results of O4 and O5. However, given the strong evidence of relevant TB activity in several plants and the commercial product NeemAzal, bio-guided chemical characterization of the remedies, which was not fully considered in the original plan, was initiated. This falls within O2 and is also reported here.

Finally, significant additions to the original plan were introduced during the extension period (months 48-54) which are based on discoveries made during the 2nd reporting period and which constitute new lines of research. These can be summarized into a new objective:

O6. To characterize the TB potential of antibiotics and anti-HIV drugs in in vitro cultures and mosquito infections, respectively.

Therefore, O6 introduced two new deliverables: 37 (4D6), planned to be disseminated through reporting to project participants by the end of the project, and 38 (4D7), planned to produce a manuscript submitted to publication by the end of the project.

O1-2 (4D2). Thousands of known schizonticidal molecules have been screened and characterized in the TB assays covering different aspects of gametocyte-to-oocyst development. 550 compounds out of 13500 tested in the *P. berghei* ookinete development assay have shown >50% inhibitory

activity in this assay at 1µM. Seventeen out of 25 compounds tested in the *P. falciparum* exflagellation assay showed > 50% inhibition at 1µM, and 10 out of 27 compounds showed transmission blocking activity in the *P. berghei* transmission assay inhibiting oocyst numbers by > 50% at 10 µM. A tool compound designed to selectively inhibit calcium dependent protein kinase 4 (CDPK4) blocked transmission in vivo when administered to mice at 10 mg/kg. The complete but transient effect validated CDPK4 as a transmission blocking target and highlighted the need to improve pharmacokinetic properties. 13k compounds were screened against the enzyme and a high proportion of hits (>10 compounds) were shown to block transmission in a membrane feeding assay. Also an additional 23 plant extracts were tested in the ookinete assay, of these 8 were found active inhibiting ookinete development by > 80% at 50µg/ml. The most active, showing a 50% inhibitory concentration < 20 ug/ml were NeemAzal® (IC50 7.72 ± 0.47ug/ml), an AcOEt fraction of neem fruits (IC50 11.58 ± 1.51ug/ml) and a *Khaya-Alstonia* ethanolic extract (IC50 10.77 ± 1.19ug/ml).

Malaria coincides with HIV infections in Africa and, recently, the antimalarial potential of anti-HIV drugs received great attention. We tested the effect of 5 out of 10 HIV-PrIs currently in use (nelfinavir, saquinavir, lopinavir, ritonavir and indinavir) on *P. berghei* gametocyte to ookinete development in *in vitro* cultures various assays. The results showed that high concentrations (100 µM) of nelfinavir affect male gamete exflagellation, while lopinavir, ritonavir and nelfinavir affect significantly the zygote-to-ookinete development at 20µM concentrations. We are now testing the 5 HIV-PrIs in gametocyte membrane feedings, in order to validate their stability and effectiveness in the mosquito midgut environment.Data from these activities have been published in (Chianese et al., 2010; Delves et al., 2012; Delves and Sinden, 2010; Lucantoni et al., 2010; Slavic et al., 2011; Tepongning et al., 2011).

O3 (4M2, 4D3). NeemAzal® was revealed to affect the vector itself. When administered to *A. stephensi* by repeated blood meals on treated mice it was found to reduce in a dose dependent manner the mosquito capacity to feed, oviposition and hatchability of eggs. At the dose of 100 mg/kg mouse and after the 3rd blood meal, mosquito feeding was found to be reduced by 50% and oviposition and egg hatchability by 60% respectively. However, no effect on mosquito survival was noticed ([T5]**[1]**. Gametocytocidal activity was assessed *in vivo* for NeemAzal® and IRAB, a purified ethanol fraction from neem leaves (gametocytocidal *in vitro*). Administering the products in a prophylactic scheme (*P.yoelii* model) at daily doses of 50 mg/kg and 25 mg/ml to mice for 15 days and challenging the animals by infected mosquito bites on day 8 of treatment, did reduce parasitemia and gametocytemia by 60% (day 15) in both treatment groups; however gametocytes, were not found to be suppressed specifically.

O4 (4M3, 4D4). Plant extracts and the commercial product NeemAzal®, which proved to possess significant anti-ESS (Early Sporogonic Stages) activities in ookinete development assays, were dose ranged to assess IC_{50}, IC_{25}, $IC_{12.5}$ and $IC_{6.5}$ values. Various combinations were then explored for synergistic effects, namely NeemAzal® with *Vernonia amygdalina, Khaya ivorensis*, *Azadirachta indica* epicarp and *Guiera senegalensis* preparations. Tests consistently evidenced additional but not synergistic effects. For example, in a NA – *V. amygdalina* replicate experiment, NA tested at 6.5 µg/ ml resulted in a 65.8% ESS development inhibition, *V. amygdalina* at 6,25 µg/ ml inhibited ESS development by 31.5% inhibition and the combination at 12.5 µg/ ml (1:1) by 86 % (**Table 4.1**).

O5 (4M3, 4D4, 4D5). TB studies with NeemAzal® on *P. falciparum* field isolates showed a complete block of oocyst development when added to artificial gametocytemic blood feeds at 70 ppm. Still, at 60 ppm complete blockages of transmission was observed in 4 out of 5 and at 50 ppm in 2 out of 5 replicate experiments (see 2nd periodic report). Based on these results the additional benefit of NeemAzal® (envisaged as a possible post-treatment strategy after ACT treatment) was assessed. Blood samples were taken from gametocytemic children before (day 0) and after a 3-day treatment course (day 4) with artesunate amodiaquine (ASAQ) and administrated to *A. coluzzii* (aka *A. gambiae* M form) females by membrane feeding. Aliquots of day 4 post ACT blood samples were treated with NeemAzal at 50 or 60 ppm. Gametocyte infectivity was evaluated by assessing oocyst prevalence and density on mosquito midguts. Replicate experiments were conducted with 9 different blood donors.

Gametocytemia varied from 48-288 gametocytes/ul blood on day 0 and similarly from 16-224 gametocytes/ul on day 4 after ACT treatment. An oocyst prevalence of 42% (CI95 23-60) and density of 10.78 (CI95 0.0-21.9) was still found after ASAQ treatment, not different from pre-treatment values (density 9.5 [CI95 0.0-19.6]) with oocyst prevalence of 59% (CI_{95} 43-74). However, NeemAzal® added to ACT post-treatment gametocytaemic blood completely blocked oocyst development at 60 ppm and at 50 ppm in 6 out of 9 replicate feeds did not yield any positive mosquito. Of the 8 positive mosquitoes in total observed in the 50 ppm treatment groups, 7 had only a single oocyst.

Table 4.1 – Inhibition of ESS by plant extracts and their combinations

Assay		**DMSO**	**NeemAzal®**			**Vernonia**			**NeemAzal+Vernonia**		
Conc.			**12.5**	**6.5**	**3.125**	**12.5**	**6.5**	**3.125**	**12.5**	**6.5**	**3.125**
1	ESS	49.7	9.0	17.0	36.7	27.0	34.0	43.3	7.0	27.3	51.3
	NH		81.9	65.8	26.2	45.6	31.5	12.8	85.9	45.0	0
2	ESS	148.2	10.7	40.7	128.0	82.0	115.3	110.7	24.0	103.7	109.0
	INH		92.8	72.6	13.6	44.7	22.2	25.3	83.8	30.0	26.4

The concentrations (Conc.) of NeemAzal (NA), Vernonia (VER) and their 1:1 combination are in mcg/ml. Percent (%) inhibition (INH) of Early Sporogonic Stages (ESS) is indicated.

O2 extension (addition to 4D2). The aforementioned results further support the idea of developing a transmission blocking post-treatment scheme based on *Azadirachta indica*, which could complement current therapies such as ACT. These data highlighted the need to determine the limonoid component(s) of NeemAzal®, besides azadirachtin A, that may contribute to its TB activity and examine whether bioavailability of the extract and its major active components would satisfy the druggability criteria.

NeemAzal® technical grade (NA, Trifolio-M, Lahnau, Germany) contains azadirachtin A (az A) at 34%, azadirachtin B-K at 18% and other limonoids are present at 6%. Fractionation of NeemAzal® by preparative chromatography using appropriate solvents allowed to isolate pure az A and to obtain 14 fractions among which fraction 6 is constituted by azadirachtins A to K (rel. small amount of az A) and fraction 9 contains dominantly other limonoids (rel. small amount of az A). Testing the fractions at 50µg/ml in the ookinete development assay (ODA) for activity against ESS, revealed strong inhibitory activity (88%; CI_{95} 84.5-92.2) of fraction 6, indicating that other azadirachtins besides az A may exhibit TB effects on ESS. In addition, the almost complete ESS suppression (94%; 89.8-99.0) observed in wells incubated with fraction 9 suggests anti-ESS properties also of other non-azadirachtin limonoids (**Figure 4.1**). These results were supported by dose range experiments conducted with NeemAzal® and pure az A. While NeemAzal® containing az A at 4.8 $\pm$3.2 µg/ml led to 50% ESS inhibition, double the amount pure az A was required to achieve the same level of inhibition (IC_{50} 12.6 $\pm$1.5 µg/ml; Figure 4.2).

The presence of other azadirachtins and limonoids appears to improve the bioavailability of NeemAza® compared to pure az A. Administrating the commercial product and the pure compound i.p. to Pb infected, gametocytaemic mice at az A dosages of 50 and 100 mg/kg yielded distinct bioavailability dynamics in the *ex-vivo* exflagellation assay. At 50 mg/kg, NeemAza® showed a maximum inhibition of 60 -70% 40-50min after administration, az A didn't reveal activity at this dosage. At 100 mg/ml az A showed a moderate (55-65% inhibition) and short activity peak at 40-60 min, whereas with NeemAza® at the same az A dosage a fast onset of and prolonged, almost 100% inhibition of exflagellation was noted. A moderate inhibitory activity (about 60%) was measured with NeemAzal® 100 mg treated blood up to 7 hours after product administration.

In blood samples from NeemAza® treated gametocytaemic mice, a reduced RBC aggregation around exflagellating micro-gametocytes was noted. This might be explained by an altered molecule expression on the surface of the exflagellating micro-gametocytes. Investigations on

whether the phenomenon is related to TB mechanisms of action related to the formation of viable microgametes and/or fecundity of macro-gametes are worth to be undertaken.

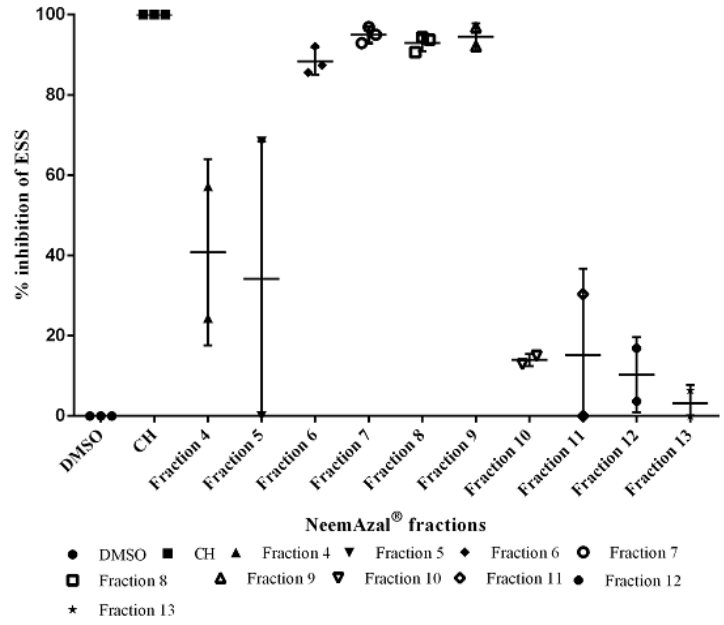

Figure 4.1 – Impact of NA fractions on ESS development *in vitro* tested at 50 µg/ml. The data points denote mean values of % inhibitions acquired from several experimental replicates. For fractions 6, 7 and 8 the data was obtained from three experimental replicates while for the remaining fractions, the data was acquired from two independent experimental replicates. DMSO, Dimethylsulphoxide, negative control; CH; Cycloheximide, positive control.

Given the various evidence of TB activity accumulated with extracts from *Guiera senegalensis* and *Vernonia amygdalina* extracts, bio-guided fractionation studies were undertaken with the aim of characterizing TB effects at the molecular level. Testing *G. senegalensis* gall fractions allowed to attribute the TB activity to molecules present in a single fraction rich in tetramethoxylated flavenoids obtained by column-chromatography fractionation of the ethylacetate phase. At 50ug/ml a 66% to 88% inhibition of ESS development was observed in the *in vitro* ookinete development assay. Interestingly, also one of the fractions derived from column chromatography separation of *G. senegalensis* stem material (ethylacetate phase) yielded prominent activity (84 - 95% inhibition). However, NMR spectra analysis suggests the presence of a large amount of different molecules in this fraction, which makes it a challenge to isolate the active ones.

Studies on *V. amygdalina* ethylacetate phase fractions obtained by column chromatography separation showed 4 fractions (fraction n.11-14 containing relatively highly polar components) capable of inhibiting almost completely (93% - 100%) ESS development at 50µg/ml. NMR spectra suggest that these fractions are rich in sesquiterpene lactones, fraction n.11 containing mainly vernolepin and fraction n.14 saponins as additional prominent components. Ongoing chemical analysis will hopefully lead to the chemical identification of the TB active molecules.

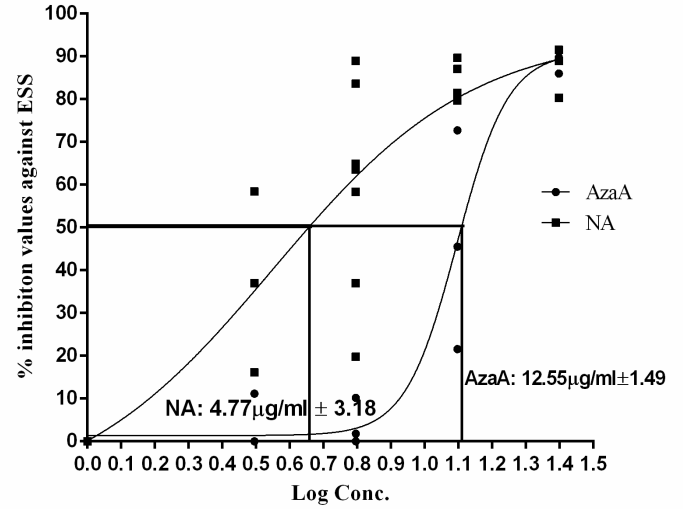

Figure 4.2 – Impact of NA and Az A on ESS development. NA and az A are tested *in vitro* at doses ranging from 3.1 – 25 µg azadirachtin A /ml. IC_{50} values were determined from 4 independent replicates, each time the concentrations tested in sextuplicate wells. NA = NeemAzal®; AzaA = azadirachtin A

Previous tests effected with *V. amygdalina* leave methanol extract indicated 15.4 µg/ml (CI_{95}: 12.7 - 18.8 µg/ml) as the concentration of the crude extract to obtain a 50% inhibition on ESS development. Stage specific counts at the fluorescent microscope allowed to reveal a differential impact on zygote formation and ookinete maturation; the capacity to interfere with the formation of banana shaped ookinetes increased with the dosage. At 50 ug/ml only one fourth of the zygotes were able to initiate the elongation process whereas in control wells 90-95% of fully matured ookinetes can be observed. These results suggest that different TB molecules may be present acting on various phases of ESS development with possibly different mechanisms of action. Isolation and chemical identification of the single molecules will allow to clarify these interesting questions.

O6 (4D6). Previous studies showed direct anti-plasmodial activities on pre-erythrocytic parasite stages of compounds designed to compete for the active site of the HIV-1 aspartyl protease (HIV-PIs) or a strong combinatorial effect with currently used antimalarial drugs. However, the transmission blocking potential of HIV-PIs has been unexplored. Three of the 10 HIV-PIs currently in use, including nelfinavir (NFV), lopinavir (LPV) and ritonavir (RTV), were tested on *P. berghei* gametocyte to ookinete development in in vitro culture assays. Mosquito infections are ongoing.

Using axenic *P. berghei* zygote/ookinete cultures we recorded a range of dose-dependent and distinct “chemotypes” (through morphological criteria and molecular markers) induced upon treatment with three HIV-PIs’, Lopinavir (LPV), Ritonavir (RTV), and Nelfinavir (NFV). Previous studies of HIV-PIs’ effects against malaria parasites revealed LPV as the most suitable effector (independently or synergistically with RTV) against the asexual forms of a number of *P. falciparum* strains in vitro, and the pre-erythrocytic (liver) stage forms of murine malaria model plasmodia in vitro and in vivo. NFV showed only a limited effect against hepatic and blood stages and at relatively high concentrations. However, in our studies all three HIV PIs tested showed the same potency in inducing strong developmental arrest at the early zygotic stages (verified morphologically and through membrane feedings). Importantly, TEM inspection revealed a unique NFV “chemotype” identified by elongated nuclei with characteristic “cornea”, less electron-dense nuclear matrix, and aggregation of heterochromatin in characteristic spots.

Thus our results in *P. berghei* zygotes/ookinetes combined with the finding that LPV and RTV reduce the number of the in vitro differentiated P. falciparum gametocytes, suggest a potency of HIV-PIs as Transmission Blocking (TB) lead compounds.

The results related to the HIV-PIs effects over *P. berghei* zygotes/ookinetes have been independently presented in the 2013 BioMalPar Annual Conference. Together with the results with ES and other potential ERAD inhibitors were presented in the 4th Annual Meeting of COST CM081; New Drugs for Neglected Diseases (September 19-21 2012, held at Kolymbari/Crete).

O6 (4D7). It has been recently shown that the mosquito gut microbiota plays important roles in mosquito infections with malaria parasites, by either directly affecting the parasite development in the midgut or indirectly by triggering the mosquito immune system. In this context, we hypothesized that the presence of antibiotics in the bloodmeal could interfere with the bacterial proliferation, enhancing mosquito susceptibility to Plasmodium and promoting malaria transmission. Such an effect could considerably influence the prevalence of malaria in sub-Saharan Africa where the use of antibiotics is high and in some cases deployed by control and elimination programs of communicable diseases. In the context of this project, we carried out proof-of-concept experiments: we mimicked antibiotic treatment by supplementing blood with therapeutic concentrations of antibiotics and analyzed the effect of the antibiotic-containing bloodmeal on the microbiota and on components of the vectorial capacity of the mosquito *A. gambiae*.

We showed that the presence of antibiotics at therapeutic concentration in ingested human blood disturbs the mosquito gut ecosystem and positively impacts on additional parameters influencing the mosquito capacity to transmit malaria, including mosquito susceptibility to *P. falciparum* (including both in vitro cultured and sampled from infected children), survival and fecundity. If confirmed in full field settings, such influences of antibiotics on malaria transmission would be of particular importance in regions where malaria co-exists with antibiotic-treated infectious diseases. A manuscript reporting these important data has been submitted for publication (Gendrin et al.,

submitted).

We found that the effect of antibiotics is sometimes influenced by the presence of antibiotic resistant bacteria, which following ingestion of antibiotic treated bloodmeal become dominant due to the lack of colonisation resistance. One such bacterium is the endosymbiotic multidrug resistant *Elizabethkingia meningoseptica*. We showed that large numbers of this bacterium are directly associated with a fat body melanotic pathology phenotype (Akhouayri et al., 2013). Transfer of melanised tissues into the hemolymph of healthy adult mosquitoes or direct hemolymph inoculation with isolated *E. meningoseptica* were the only means for transmission and *de novo* formation of melanotic lesions, specifically in the fat body tissues of recipient individuals. We show that *E. meningoseptica* can be vertically transmitted from eggs to larvae and that *E. meningoseptica*-mono-associated mosquitoes display significant mortality, which is further enhanced upon *Plasmodium* infection, suggesting a synergistic impact of *E. meningoseptica* and *Plasmodium* on mosquito survival.

WP5: Transmission Blocking via Immune Mosquitoes

WP #:	5	**Months:**						1-54
WP title:	Transmission Blocking via Immune Mosquitoes							
Activity type:	RTD							
Participant #:	1	2	3	4	5	6	7	**Total**
Person months/participant:	10.8	0	41.5	53	0	0	0	**105.3**

Summary: We established assays to test a series of native or bio-engineered serpins for their inhibitory potential against parasitic proteases and the sporogonic development of *P. berghei*. We also developed a set of vectors for the conditional expression of transgenes in the midgut, as well as for direct expression of LRIM1 in the fat body. The latter vector contained two PhiC31 docking sites to allow *in vivo* cassette exchange of LRIM1 for other effector genes including APLC1. Five native or engineered mutant variants of serpins were tested for potential anti-plasmodial activity using three validated *in vitro* and *in vivo* assays. None of the inhibitors displayed significant inhibitory potential to warrant production of transgenics lines overexpressing these candidates. However, *in vivo* silencing and characterisation of endogenous matrix-metalloproteases and their single inhibitor TIMP established that MMP1 protects *Plasmodium* from gradual elimination at the oocyst stage, and therefore suggests that overexpression of the putative inhibitor of MMP1 in transgenic mosquitoes may be a means to malaria refractoriness. Phenotypic characterisation of MMP1, however, did not confirm our hypothesis and plans to produce transgenic mosquitoes were abandoned. Instead we focused in overexpression of the complement factors LRIM1/APL1C, which antagonise ookinetes by delivering the opsonin TEP1 on their surface leading to parasite lysis. The simultaneous expression of two genes presented a major technological challenge, which we successfully accomplished. We produced a number of transgenic lines expressing the LRIM1/APL1C complex in the hemolymph, which are now tested for their capacity to block or reduce parasite infection and hence affect malaria transmission.

Objectives:

O1. Screen a number of naturally derived or molecular engineered protease inhibitors and identify the most potent transmission blocking agents;

O2. Generate transgenic *A. gambiae* lines over-expressing known immune regulators of parasite development (e.g. *TEP1R*, *LRIM1*, *APL1C*, and *REL2F*) and peptides identified through O1 in a gut-specific manner;

O3. Generate transgenic *A. gambiae* lines over-expressing lines inducing fat body specific knockdown (kd) of CACTUS;

O4. Examine and compare the malaria transmission blocking potential of GM mosquitoes; and

O5. Examine the fitness costs and benefits associated with GM and infection.

Modifications: Recommendations made by the first EVEC to reduce the aims and careful consideration of the technical difficulties and effort to generate and maintain transgenic lines as well as recent developments in the field, led to the following modifications: **O1** was extended to *in vitro* (to improve expression) and *in vivo* (to monitor effect on P. falciparum development) analysis before moving to transgenic mosquitoes overexpressing NcPIs; **O2** was modified to incorporate 1) the development of conditional (Gal4-UAS) expression systems for the midgut and 2) testing of TIMP, the only inhibitor of MMPs present in *A. gambiae*; **O3** was modified to include *LRIM1 and APL1C* overexpression in the fat body using the PhiC31 integrase for site directed insertion and exchange of these LRR genes, rather than CACTUS, as the REL1/CACTUS pathway is shown not to have an effect on *P. falciparum*.

O1. The initial workplan focused on the testing of purified protease inhibitors to block *Plasmodium* development. This involved expression of recombinant inhibitor proteins and establishment of reliable assays to quantify their activity against sporogonic development and protease activity. These assays initially included i) membrane feeding of gametocyte- and/or purified ookinete meals with recombinant serpins included and ii) co-culture of ookinetes with insect cells transiently expressing modified serpins. We have further developed assays that tested inhibitory potential following injection into the hemolymph of *P. berghei* infected mosquitoes. In total, five native or engineered mutant variants of serpins were tested for potential anti-plasmodial activity using the three validated assays **(5M1)**. The combined data from these experiments discouraged further efforts to take these candidates forward for transgenic mosquito creation. A further candidate, TIMP, has shown greater promise, since *in vivo* gene silencing experiments have indicated a clear role for MMP1 in oocyst development. MMP1 association with early oocyst surface suggests that MMP1 is involved with either oocyst protection or oocyst development. TIMP is the only known endogenous inhibitor of metalloproteases and is now taken forward for conditional overexpression in transgenic *An. gambiae* fat body (**5M2, 5D1**).

O2. The modified workplan for O2 outlined above involved 1) the development of a conditional expression system and 2) the overexpression of TIMP, both in a gut specific manner in transgenic *An. gambiae*. A number of different vectors have been constructed that will regulate expression of target immunity genes and selected reporter genes in transgenic mosquito lines. We generated and characterized transgenic mosquitoes expressing epitope-tagged LRIM1 in the fat body following blood meal (now also utilized in O3) and conditionally expressed transgenes in the midgut **(5M3)**. To examine the Gal4-UAS system *in vivo*, transgenic An. gambiae driver lines carrying a modified Gal4 gene under the control of the carboxypeptidase promoter, and responder lines carrying UAS regulated luciferase and eYFP reporter genes were created. Crossing of the Gal4 and UAS lines resulted in progeny that expressed both reporters in the expected midgut specific pattern. The midgut driver and dual reporter responder constructs are the first to be developed and tested successfully in transgenic An. gambiae and provide the basis for expression of further transmission blocking genes in the mosquito **(5.M4, 5.D2)** (Lynd and Lycett, 2012). We have also tested the function of the Matrix Metalloprotease 1 (MMP1), which is present in two forms expressed in hemocytes and the midgut, respectively, and its putative Tissue Inhibitor of Metalloproteases (TIMP) that is expressed in the midgut. RNAi-mediated silencing of *MMP1* drastically reduced the number of developing oocysts, but without affecting the midgut invasion process *per se*, suggesting a likely post-invasion protective function. However, RNAi silencing of *TIMP* had no apparent effect on mosquito midgut invasion and/or on oocyst growth. Therefore, further transgenic overexpression of TIMP was unnecessary.

O3. The modified workplan for objective 3 outlined above was targeted towards the generation of transgenic lines that overexpress the LRIM1 and APLC1 effector genes; known antagonists of *Plasmodium* transmission. Initially a series of LRIM1 expressing transgenic lines were created which also included dual phiC31 docking sites to allow APL1C integration or exchange into the same localization. Six lines produced secreted tagged LRIM1 following a blood meal **(5M4)**, and the two highest expressing lines, A11 and BB57, plus an intriguing mutant (HOMO) displaying a melanized larval cuticle, were provided to Imperial College to examine TB potential **(5M5)**. We have demonstrated for the first time that cassette exchange is functional in mosquitoes. Using the A11 strain as the parental line, we have generated a series of transgenic lines that carry both

tagged LRIM1 and tagged APL1C in the same genomic locus **(5M5)**. In the same series, we have also produced lines that only contain the tagged APL1C cassette at this locus. Having the different effector transgenes in the same locus eliminates position effect, and allows direct comparison of the alternative lines for transmission blocking phenotype. Biochemical analysis indicates that the double transgenic lines express and secrete stable, tagged heterodimeric complexes of recombinant tagged LRIM1/APL1C into the hemolymph. In the single transgenic lines, tagged homodimeric complexes are detected circulating in the blood **(Figure 5.1).** Native homodimeric complexes are not observed in the hemolymph, which may suggest that there is a mechanism regulating heterocomplex formation specific to hemocytes.

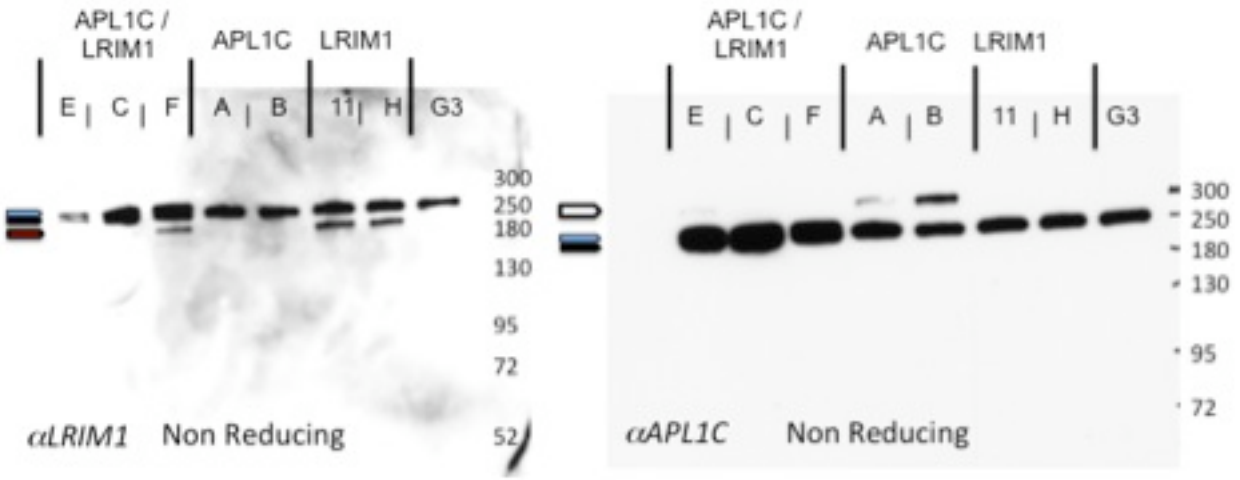

Figure 5.1 – Non reducing western blot of hemolymph extracts from isogenic transgenic lines expressing both APL1C and LRIM1 effectors (E, C, and F), single effectors; APL1C (A, B), LRIM1 (11, H) as well as control lines G3. Blots probed with anti-LRIM1 (left) and anti APL1C (right). Blue arrow – tagged heterodimers, black arrow – native heterodimers, red arrow – tagged LRIM1 heterodimer, white arrow – native homodimer.

O4. Under objective 4, GM modified lines were examined for transmission blocking potential. The assays of the GM line overexpressing LRIM1 following a bloodmeal were performed following *P. berghei* infection at IC. Not unexpectedly, the over-expression of only LRIM1 did not appear to modify the TB potential in A11 and BB57 lines (**5M5, 5D3**). Interestingly, in this 3rd reporting period we observed that the larval melanised mutant line (HOMO) was somewhat refractory to *P. berghei* infection relative to non-transgenic sibling controls (**Figure 5.2, 5M5, 5D3**). Since the refractoriness was limited to this line, it is unlikely that the altered refractoriness is related to LRIM1 expression in the adult, since expression levels appears similar to the non-refractory A11 line.

The LRIM1 A11, LRIM1/APL1C, and APL1C lines have recently been supplied to IC to analyse both *P. falciparum* and *P. berghei* infection rates (5M5, 5D3). Although the delivery has been delayed, due to issues with staff availability in IC, this TB analysis will be completed at IC in the next couple of months, beyond the termination of the transmalariabloc project, to facilitate publication.

O5. Under objective 5, once GM lines are developed, these were to be delivered **(5D4)** to WP8 to examine the fitness cost of GM and *Plasmodium* infection. Since the LRIM1 lines initially delivered were not refractory, and the fitness characterization involves considerable work that will have limited significance with these lines, it was decided to only deliver refractory lines to WP8 for further characterization.

The insertional mutant line (HOMO) proved refractory to *P. berghei* infection. Preliminary analysis of fitness at LSTM indicated that the mutant was severely compromised. Transgenic males have low fertility and the adults suffer from flight co-ordination problems. The line thus has to be maintained as heterozygotes by breeding from wild type males and arm feeding the colony. Removal of the LRIM1 gene from the homo line by cassette exchange was shown to restore

fertility and co-ordination, suggesting that the fitness costs are entirely derived from ectopic expression of LRIM1 during larval stages. Because these fitness costs were severe, the HOMO line was not delivered **(5D3)** to WP8. The refractoriness is most probably related to the fitness of the line, rather than a consequence of LRIM1 expression in the adult, and so further analysis in the context of TransMalariaBloc would be of limited significance.

The LRIM1/APL1C lines are currently being assayed for TB potential at Imperial **(5D4)**. If these lines prove refractory to *P. falciparum* this will provide the final product, which is the genetically modified mosquito.

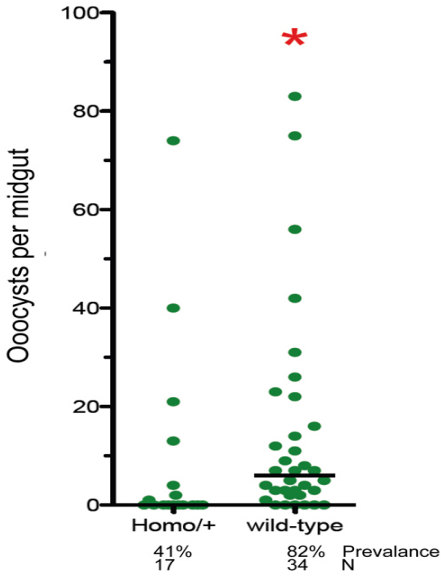

Figure 5.2 – Melanising transgenic strain (homo) displays significantly less infection than wild-type; P<0.05 Mann-Whitney.

WP6: New Transmission Blocking Targets

WP #:	6	**Months:**						1-54
WP title:	New Transmission Blocking Targets							
Activity type:	RTD							
Participant #:	1	2	3	4	5	6	7	**Total**
Person months/participant:	84	0	0	12	0	25	8	**129**

Summary: At the close of the first reporting period for WP6, we had successfully produced and validated a new hybrid microarray platform (**TASK1, M1, D1, Month 6**). Its development allowed for the simultaneous profiling of parasite and mosquito gene expression patterns. Mosquito infections with sympatric malaria parasite field isolates have been performed in Burkina Faso (**TASK2, M2, Month 18).** Also, the *A. gambiae* and *A. arabiensis* transcriptional responses to infections with *P. falciparum* populations sampled directly from infected children were profiled using DNA microarrays and RNA sequencing. In parallel, the developmental transcriptomes of these *P. falciparum* populations in the mosquito midgut were profiled at various time points. Analysis of the data revealed that mosquito responses are affected by many factors including the infection intensity, parasite origin (whether sympatric or allopatric) and parasite genotype, and that co-adaptation between vectors and parasites may act to minimize the infection impact on mosquito fitness. RNAi silencing provided initial evidence for important roles of mosquito G-protein coupled receptors (GPCRs) in controlling infection intensity-dependent anti-parasitic responses Moreover, hundreds of *P. falciparum* genes are differentially regulated during the first 24 hours after infection. Discrete transcriptional programs that regulate the various developmental events in the mosquito midgut were detected. Gene disruption in *P. berghei* led to the identification of four novel regulators of ookinete development and the ookinete to oocyst transition. Furthermore, we completed our work with analysing the responses of the *A. gambiae* and *A. arabiensis* midguts to infections with P. falciparum populations sampled from infected children in Burkina Faso. The analysis revealed

conserved as well as divergent responses between the two vectors, some of which begin already 1 h post infection. Among these was the neuropeptide MIP, silencing of which increases significantly the infection intensities, which together with our previous data reveal a very important role of GPCR signalling during infection. In addition, we finalised the analysis of the developmental profiles of *P. falciparum* inside the mosquito midgut, which revealed that these profiles are remarkably similar between the two vectors as well as between different parasite strains. This analysis together with an equivalent analysis of *P. berghei* sexual and sporogonic development led us to select and knockout several genes that we then phenotypically analysed in *A. gambiae* infections. All the genes exhibited phenotypes that are associated with mosquito midgut infection and some of them are currently explored as potential candidates for transmission blocking interventions.

Objectives: This work package aims to identify and characterize conserved mosquito and parasite genome-wide responses in order to identify ideal targets for malaria transmission blocking. All the objectives of this WP pertained to the current reporting period:

O1. Identification of mosquito molecular responses to *P. falciparum* sympatric field isolates during different stages of parasite development in the mosquito gut, in two species of mosquitoes and two distant geographic regions;

O2. Characterization of genomic expression profile of *P. falciparum* field isolates during development in the mosquito gut, from 2 distant geographic regions in different mosquito species;

O3. Identification of similarities and differences in mosquito transcriptional responses between different mosquito/parasite combinations, which may determine the susceptibility to P. falciparum;

O4. Identification of similarities and differences in *P. falciparum* expression profiles in various mosquito/parasite combinations, which may determine parasite's infectivity;

O5. Functional characterization of *A. gambiae* and *P. falciparum* genes with respect to their impact on parasite survival in the mosquito gut using information obtained from O1 and O3, and identification of new conserved targets for transmission blocking interventions.

Modifications: Due to the difficulties in establishing mosquito colonies in Uganda within a timeframe that would allow infections to be included in this project, research was focused on Burkina Faso and a second location in Central Africa (Cameroon) has been chosen for the comparative analyses of transcriptional profiles. The Iganga insectary and transmission facilities, which were completed recently, will be used in the continuation of the project.

O1-4. We generated and validated (**6M1; 6D1**) and then used a hybrid *A. gambiae*/*P. falciparum* DNA oligonucleotide microarray platform to monitor in parallel the developmental transcriptomes of *P. falciparum* populations, sampled directly from infected children in Burkina Faso, in the *A. gambiae* and *A. arabiensis* midgut and the transcriptional responses of the mosquito midgut. RNA prepared from midguts of infected mosquitoes (**6M2**) was hybridized on these microarrays (**6M3**) and ANOVA analysis was performed following various filtering protocols (**6M4**). To detect low abundance transcripts falling near the microarray detection limit we additionally used next generation RNA sequencing using the SoLID technology.

The combined results revealed hundreds of *P. falciparum* genes that are differentially regulated at 1h, 6h, 10h & 22-24h post infection. Profiling of *P. berghei* developmental transcriptomes in *A. gambiae* using an equivalent design highlighted hundreds of orthologous genes with profiles similar to those of *P. falciparum*, while comparison of these profiles with those of mutant *P. berghei* revealed genes of which the expression already starts in mature gametocytes, altogether helping us prioritizing genes for phenotypic and functional characterization (**6M5, 6D3**).

In addition, we used the same technique and protocols as above (**6M2-4**) to investigate the transcriptional response of Cameroonian *A. gambiae* to geographically related *P. falciparum* at varying infection intensities and different infection stages (Mendes et al., 2011). The results demonstrated that mosquito responses are infection intensity dependent. A major transcriptional suppression of genes involved in the regulation of midgut homeostasis was detected in low-intensity infections, suggesting that co-adaptation between vectors and parasites may act to minimize the infection impact on mosquito fitness, by selectively suppressing specific functional

classes of genes.

To identify similarities and differences between different mosquito/parasite combinations in *A. gambiae* responses to *P. falciparum*, recently colonized Burkina Faso and Cameroon mosquitoes were infected with sympatric and allopatric parasite isolates and DNA microarrays were used to register the mosquito transcriptional responses during mosquito midgut invasion, 24h after ingestion of infected blood meal (Harris et al. ASTMH meeting, and manuscript in preparation). The results revealed that mosquito transcriptional responses to infection are indeed multi-factorial: the most important factor is parasite origin, while mosquito origin plays a secondary yet important role. Infection intensity is also an important factor, consistent with both the data presented above (Mendes et al., 2011) and results in WP8 showing that sympatric combinations produce lower infection intensities (Harris et al., 2012). Importantly, the transcriptional response is highly diverse across infections independently of sympatric/allopatric combination, suggesting a strong genetic component possibly largely attributed to parasite genotypes. Nevertheless, genes exhibiting conserved responses across all infections, which are now tested for universal effects on parasite infections (**6M5, 6D2**). Our findings have great implications on the design of transmission blocking interventions. The fact that vector-parasite interactions may be differentially evolving over space and/or time indicates that any such intervention must be rigorously assessed before implementation.

O5. From the transcriptome analyses reported above, several parasite and mosquito genes were prioritized for phenotypic and functional characterization. With respect to mosquito genes, results reported above identified Rhodopsin-like G protein-coupled receptors (GPCRs) as a prominent differentially regulated gene class in *P. falciparum*-infected mosquitoes. GPCRs encode receptors that function in cell communication by bridging extracellular ligands (hormones, neurotransmitters, light and odor sensors, chemokines, etc.) with downstream intracellular effectors, thereby playing an essential role in important physiological processes such as development, reproduction, feeding behavior, diuresis, and immunity (**6D4**); therefore they are considered ideal targets for interventions targeting the vector or vector/parasite interactions. RNAi gene silencing showed that the *A. gambiae* gastrin/bombesin receptors GPGRP1 and GPGRP2 are antagonists of *P. falciparum* when infection intensities are high, whereas the putative neuropeptide Y receptor GPRNPY3 and the gastrin/cholecystokinin receptor GPRCCK1 are *P. falciparum* agonists (Mendes et al., 2011). These data highlight the importance of GPCRs in regulating *P. falciparum* infections. Targets for transmission blocking among these GPCRs and other genes are currently being selected (**6D5**).

With respect to parasite genes, 4 mutant parasites were phenotypically characterized to date and shown to dramatically affect transmission: 2 of them lead to defective ookinete development and the other 2 show normal ookinetes that are unable to develop to oocysts. Further characterization was carried out for the latter 2 genes. The first encodes a formin-like nuclear protein, MISFIT, expressed in mature male gametocytes likely affecting mitosis during gametogenesis; however its effect is manifested much later during the ookinete-to-oocyst transition (Bushell et al., 2009). We created vectors expressing various versions of MISFIT's FH2 domain, which is thought to bind actin or microtubules (MT). Recombinant proteins will be used in structural studies for compound positioning and development of an assay for compound screening based on actin or MT binding. The second gene encodes a putative subtilysin-type secreted protease, SUBO, which is specifically expressed in ookinetes and localized on the ookinete membrane. In addition, antibodies have been raised against proteins encoded by all four genes, which are currently being tested for transmission blocking activity (**6D5**).

To streamline our phenotypic analysis pipeline of *P. berghei* mutants and enable studies of parasite-mosquito genotype*genotype interactions, we developed and validated a novel *P. berghei-A. gambiae* dual co-infection system that consists of a GFP-expressing parasite line lacking candidate genes of interest, a red fluorescence parasite line expressing mCherry that is used to calibrate the GFP parasite infectivity in co-infection experiments, a wild-type mosquito line that is used for infections (or as a reference in genotype*genotype studies), and a mosquito line silenced for candidate mosquito candidate genes factors (optional, for genotype*genotype studies). This methodological breakthrough addresses current hindrances in quantitative characterization of parasite developmental phenotypes in the vector and enables investigation of mosquito-parasite

g*g interactions.

All the milestones and deliverables but **6D5** had been met at the close of the second reporting period; however, our work to characterize additional TB targets or in more depth already identified targets continued in the third reporting period. These as well as the accomplishment of the outstanding deliverable 6D5 are reported here. Within this period, an additional manuscript reporting data related to parasite targets has been submitted (Akinosoglou, Bushell, Ukegbu et al., submitted), while two manuscripts reporting data on mosquito targets (**6D5**) are in preparation. A final year thesis reporting on deliverable 6D5 has been presented (Trasanidis, 2013), while one final year (Taxiarchi, 2013) and two MRes (Cho 2011, Seles 2013) theses reporting on activities of this WP related to the parasite work were presented.

The comparative analysis of the *A. gambiae* and *A. arabiensis* midgut transcriptomes at four distinct timepoints after infection (1h, 6h, 10h and 24h post-infection) revealed significant inter-species as well as species-specific transcriptional responses. One of the genes that is induced highly soon after infection is Neuropeptide M or Myoinhibitory peptide (MIP) (**Figure 6.1**)**.** RNA interference (RNAi)-mediated gene silencing illuminated a significant role of MIP; its knockdown increased significantly the number of oocysts in the mosquito midgut, both *P. berghei* and laboratory *P. falciparum*. MIP encodes a beta-type allatostatin and is known to inhibit the production of Juvenile Hormone (JH) through GPCR signalling. JH is very important for mosquito development and reproduction and reduces food uptake by inhibiting gut muscle contraction. These data in conjunction with data reported previously on the role of GPCR signalling in controlling the intensity of *Plasmodium* infection identify a very important interplay between the mosquito behaviour and Plasmodium infection, which can result in the development of new targets for malaria transmission blocking (**6D5**). On the one hand, GPCRs bind extracellular ligands and control physiology and represent >40% of all the current drug targets. Therefore, they can be exploited towards development of transmission blocking drugs. On the other hand, MIP and other neuropeptides are short and immunogenic peptides secreted early after bloodmeal and infection and can therefore be considered good candidates for transmission blocking vaccines.

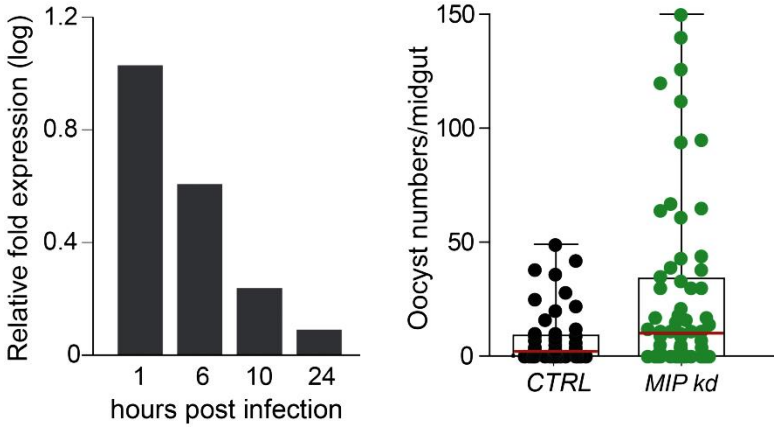

Figure 6.1 – MIP plays an important role in *Plasmodium* infection. Left panel, MIP expression in the mosquito midgut is induced as early as 1 h post infection. Right panel, RNAi gene silencing of MIP in *A. gambiae* increases the numbers of *P. berghei* oocysts in the mosquito midgut.

In parallel with profiling the responses of the *A. gambiae* and *A. arabiensis* midgut, we characterised the developmental transcriptomes *of P. falciparum*. In parallel, this work has also characterized and identified the mosquito molecular responses to *P. falciparum* sympatric field isolates in the two species of mosquitoes. This analysis has shown that in contrast to the mosquito response which presents significant differences between the two vectors, the parasite transcriptional profiles are strikingly almost identical between the two vectors. This is likely due to the rapid and precise developmental programme that the parasite must complete during these first 24 hours in the vector. We selected five *P. falciparum* candidate genes that have orthologs in *P. berghei* since gene knockouts and mutant developmental characterization are carried out in the *P. berghei*. These genes were designated as Pfc01, Pfc57, Pfc43, Pfc53 and Pfc22. The first three genes encode secreted or membrane bound proteins and can be therefore suitable for transmission blocking vaccines. Generation of P. berghei ko mutants and phenotypic analysis of these mutants in mosquito infections revealed important roles of all these genes in mosquito midgut infection; ko of Pfc01, Pfc22, Pfc53 and Pfc57 leads to defective epithelium invasion, while

knockout of Pfc43 results in ookinetes that successfully cross the epithelium but are killed while exiting the midgut epithelium.

We also profiled the transcriptomes of the rodent malaria parasite *P. berghei in vivo*, in the midgut of *A. gambiae* mosquitoes (Akinosoglou, Bushell, Ukegbu et al., submitted). Data analysis revealed distinct transcriptional programs putatively involved in developmental processes or interactions with the mosquito. Targeted disruption of three genes resulted in parasites exhibiting significant midgut infection phenotypes. Loss of the peptide GAMER impairs male gamete release, while knockout of the putative Magnesium phosphatase HADO affects ookinete development. An intriguing discovery was SUBO, an ookinete-specific subtilisin localized on the ookinete surface. SUBO loss-of-function leads to morphologically normal, motile ookinetes with compromised invasion ability. The combination of the *P. falciparum* and *P. berghei* data provide novel insights into the molecular framework underpinning *Plasmodium* development in the mosquito and identifies targets for malaria transmission blockade (**6D3**). The P. falciparum secreted and transmembrane proteins Pfc01, Pfc57, Pfc43 and SUBO have been cloned and stably expressed, and antibodies are currently being produced.

*WP7: Genes and genotype*genotype interactions*

WP #:	7	**Months:**						1-54
WP title:	Genes and genotype*genotype interactions							
Activity type:	RTD							
Participant #:	1	2	3	4	5	6	7	**Total**
Person months/participant:	73.47	0	0	78	2	25	58.05	**236.52**

Summary: We developed an Affymetrix custom single nucleotide polymorphism (SNP) genotyping array for analysis of 400,000 SNPs identified through sequencing of the M and S incipient species of *A. gambiae*. We used this SNP chip to interrogate a laboratory colony of M form A. gambiae with regards to their susceptibility to infections with laboratory NF54 *P. falciparum*. This work led to the discovery of several genomic regions containing a number of genes that could contribute to the observed phenotype. While the phenotypic characterisation of these genes is on-going, we made a technological leap toward directly identifying the SNPs and genes causing the infection phenotype by carrying out whole genome sequencing of individuals at the phenotypic extremes of infection levels in both M and S form populations colonised in Burkina Faso and infected with *P. falciparum* from local gametocyte carriers. These two whole genome sequencing projects have now been completed and we are currently in the final phases of candidate confirmation using independent infections and RNAi knockdown of candidate genes. We also used the SNP chip to identify *A. gambiae* alleles that are involved in the interactions with gut bacteria and thus are likely to affect parasite infection. Our analysis revealed a family of fribronectin type III domain proteins that are involved in the homeostasis of Enterobacteriaceae, as well as a gustatory receptor that seems to control satiation circuits that influence bacterial ingestion.

Objectives:

O1. Identify loci harboring natural variation that contributes to susceptibility to malaria infection in two species of mosquito from two geographic regions;

O2. Identify loci harboring natural variation that contributes to infectivity in *P. falciparum* using multiple isolates from two geographic regions;

O3. Using information gathered from objectives 1 and 2 (completed), characterize the mechanisms of natural refractoriness to infection;

O4. Evaluate the importance of host genotype*parasite genotype interactions (for each mosquito species, parasite isolate, and geographic region) on mosquito susceptibility and *P. falciparum* infectivity.

O5. Process population biology data and include them in a population biology sub- database to be

included in VectorBase. Development of linked ontologies-controlled vocabularies to handle the population biology data.

Modifications: As discussed in the previous reporting period, development of a database to handle mosquito population genetic data is now handled by and reported in WP7 for consistency. The original deliverables **2D2** of WP2 and **7D4** of WP7, which refer to the same database resource, are now merged into one deliverable referred to as **2D2&7D4**. In addition and as discussed below, we successfully developed and made available our Affymetrix 400,000 SNPchip **(7D2, M2).** Because this milestone **(M2)** was fully met, we additionally explored the use of whole genome sequencing of individuals at the phenotypic extremes of infection levels to complement and augment the SNP chip results.

In the beginning of the project various modifications were made to the research plan of WP7 that take into account technological advances and new knowledge, which facilitate achievement of the objectives that remain identical or enhanced relative to those originally proposed. These include: (a) the construction of an Affymetrix 400,000 SNPchip instead of the IIllumina's Golden Gate Platform, that would allow us to interrogate only 1536 SNPs; (b) optimisation of a protocol that uses pooled instead of individual mosquitoes, which maximises throughput and minimises costs; and (3) use of mosquito colonies rather than isofemale lines, made possible by the advanced SNPchip technological platform that allows assaying at distances close to the observable linkage disequilibrium (LD) in *A. gambiae* (see below).

Colony generation and experimental design: Colonies of *A. gambiae* M and S and *A. arabiensis* were generated in Burkina Faso and are reported in WP8. Although colonies are outbred, and founded from numerous mated females collected in nature, low but observable LD (the non-random association of alleles at two or more genomic loci) exists due in part to bottlenecking during lab adaptation. In natural populations, average LD is less than 300bp, the median distance between SNPs on our array. The slightly increased LD in colonies relative to nature combined with still abundant genetic variation offers power to the colony method that in effect serves as a combined QTL and association mapping analysis.

SNP identification and SNPchip construction: In collaboration with N. Besansky at the University of Notre Dame, the Venter Institute and the Genome Centre at WashU, *A. gambiae* colonies of M and S established in 2005 from Mali were genome sequenced. Both colonies were homosequential and homozygous with respect to all known chromosomal inversions with the exception of 2L*a* and 2R*c*. Independent draft genome assemblies were generated based on ~2.7 million Sanger whole-genome shotgun reads. Assemblies were performed independently of the reference *A. gambiae* PEST genome, a chimera of M and S. Genome assembly metrics were similar between M and S. Lower average read coverage contributed to assembly gaps, motivating alignment of the M and S scaffolds to the PEST assembly for transfer of genomic coordinates and gene annotations. From these sequences, several million SNPs segregating within and between M and S, and additional sites fixed between them were annotated.

Based on the M/S genome sequences, we developed a SNPchip in the Affymetrix array 49-format to interrogate *A. gambiae* allelic variation at 399,713 genomic positions (plus some hundreds of *P. falciparum* SNPs that will allow us to genotype *Plasmodium* populations) using up to seven pairs of perfect-match, offset probes per SNP. RMA background subtraction and quantile normalization using Affymetrix Power Tools was used to call individual SNP genotypes (A/A, A/B, or B/B) using the BRLMM-P software. Alternatively, the relative frequency of the A allele among pooled DNA samples was calculated when DNA from multiple individuals was pooled. We hybridized individual arrays with genomic DNA from each of 20 field-collected females from the three known sympatric *A. gambiae* populations in Mali (M, S, and Bamako) that exhibit partial reproductive isolation. We then hybridized DNA pooled from the same 20 females from each population to determine the degree to which quantitative differences in allele frequencies could be assessed using pooled DNA. We also hybridized a pool of DNA from 20 field-collected individuals of the sister species *A. arabiensis*. Results obtained from pooled and individual hybridizations were highly correlated (Pearson's r^2 = 0.96 for M, S, and Bamako comparisons), indicating that the majority of SNPs assayed on the array yield useful quantitative information regarding divergence in allele frequencies between pooled samples.

Genotyping: As a proof-of-concept to identify genetic variation that affects mosquito infections with *Plasmodium*, we used laboratory *P. falciparum* NF54 infected blood that was fed to nearly 500 females from a recently established Cameroonian colony of M-form *A. gambiae*. Infection intensities varied from fully refractory to nearly 500 oocysts per midgut. We chose three pools of highly susceptible, lowly susceptible, and fully refractory mosquitoes to hybridize to the chips in two technical replicates **(Fig WP7.1A)**. We calculated the difference in contrast values between pools (High vs Low, Low vs Refractory, and High vs Refractory) using a permutation analysis. This analysis compares the summed contrast difference over 10 neighbouring SNPs to the null distribution of the summed contrast difference of 10 randomly drawn SNPs and looks for both excess differentiation in contrast as well as an excess number of sites in a given window with high differentiation. Bonferroni correction was then applied to all significant windows. Using this method, we identified several small (< 2Mb) clusters (e.g., clustered red circles on 3L of **Fig WP7.1B**) as well as some even smaller windows that contain one or two candidate genes that appear to harbour allelic variation contributing to the infection outcome.

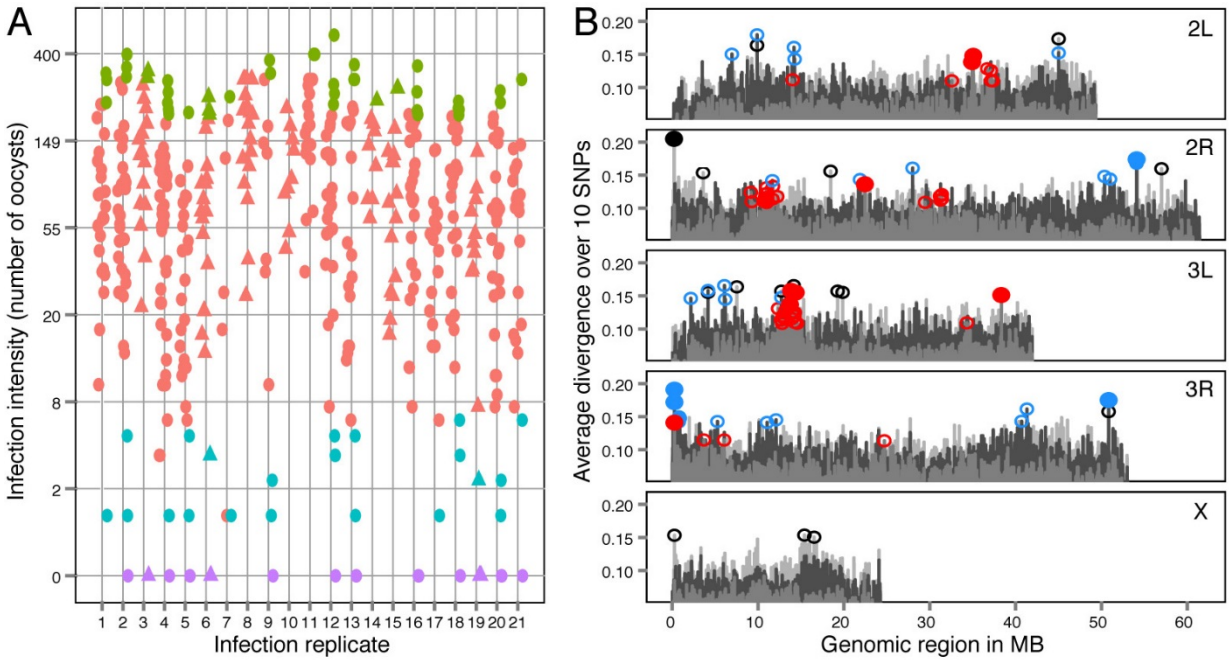

Fig WP7.1: Genomic variation affecting M-form *An. gambiae* susceptibility to *P. falciparum* using a 400K SNPchip. (A) Distribution of infection intensities across nearly 500 female mosquitoes infected with *P. falciparum* NF54 gametocyte culture. Pools are coloured as follows: Highly susceptible in green (46 individuals, 200-500 oocysts), Lowly susceptible in cyan (24 individuals, 1-5 oocysts) and Fully refractory in purple (22 individuals, 0 oocysts). A total of 402 individuals showing medium level susceptibility were excluded (red). **(B)** Differentiation in allele frequency between High vs. Low (red circles and medium grey), High vs. Refractory (blue circles and dark grey), and Low vs. Refractory (black circles and light grey) pools. Bonferroni-corrected significant 10-SNP windows are marked with filled circles (*p<0.00000125)* while a slightly less conservative set of 10-SNP windows are marked by hollow circles (*p<0.0001*).

Prior to the unpublished results we report here, the only studies investigating the genetic basis of *An. gambiae* susceptibility to *P. falciparum* used markers located roughly every 10Mb across the mosquito genome. Our method improves the marker density by orders of magnitude and, because we do not depend on isofemale lines as previous studies have, we can genetically examine fully refractory mosquitoes, of which there are many.

We successfully generated an Affymetrix genotyping chip that interrogates 400,000 SNPs in the *A. gambiae* genome (Lawniczak et al., 2010; Neafsey et al., 2010). This chip is now available to the mosquito community through Affymetrix, a valuable contribution to the vector scientific community **(7M2, 7D2)**. We also used this chip in proof-of-concept association studies using laboratory *A. gambiae* (N'gousso, Cameroon) and *P. falciparum* (NF54 reference line) strains. We had also successfully created three colonies (*A. gambiae* M and S, and *A. arabiensis*) in Burkina Faso

(7M1, 7D1). These colonies have been repeatedly infected with gametocyte positive blood from local carriers and used in experiments by WP3, WP4, WP6 and WP8. In this WP, a total of 1371 M mosquitoes were fed blood from 7 gametocyte carriers, 1084 S mosquitoes were fed blood from 5 gametocyte carriers and 659 *A. arabiensis* mosquitoes were fed blood from 5 gametocyte carriers. Infection prevalence was on average 75%, while the average infection intensity per feed was ~50 oocysts/midgut; these are remarkable achievements for infections in the field. We are currently in the process of pooling high and low infected individuals from a subset of the more successful feeds for genotyping on our chip. Given these are colony mosquitoes, we expect to have results which will narrow down candidate loci contributing to the outcome of an infection as effectively as our proof-of-principle experiment discussed in the first report. Genotyping results are expected in May 2012 **(7D3)**.

O3. We selected 12 mosquito genes that fall into genomic regions showing genetic association with susceptibility to laboratory *P. falciparum* infections to examine using RNAi. Half of these genes were chosen because they had a single SNP with very different frequency between highly infected and lowly infected individuals, and the other half of the genes were chosen because they fell in a highly significant 10 SNP window in the permutation-based analyses. Thus far, these genes were tested only for the impact of their knockdown on *P. berghei* infection. Upon knockdown, three genes showed significantly ($p<0.001$) higher numbers of oocysts than controls and one gene showed significantly fewer oocysts than controls.

O4. This objective has been partly addressed with the analysis of sympatric vs. allopatric infections of A. gambiae M mosquitoes – see WP8 (Harris et al., 2012). The results from the experiments described above in O1-2 will be important in advancing our understanding of potential genotype*genotype interactions.

During the last reporting period, various modifications were again made to the research plan of WP7 that take into account technological advances in the field of genome sequencing and GWAS. In addition to our Affymetrix 400,000 SNPchip we also investigated successfully the use of whole genome sequencing of individuals at the phenotypic extremes of infection levels **(7D2)**. This has led to some delays in publication of results, but at the great advantage of getting directly at associated SNPs rather than having to return to sequencing in order to confirm candidate associations from the original samples.

O1-4. We have examined the underlying genetics of infection susceptibility in M and S colonies created in Burkina Faso using whole genome sequencing rather than the SNPchip we intended to use in the last reporting period. Two whole genome sequencing projects have now been completed and are in the final phases of candidate confirmation using independent infections and RNAi knockdown of candidate genes. Here we report the findings of these two projects.

We acquired further funding to carry out whole genome sequencing by writing small collaborative proposals with two other large collaborative organizations. Our *A. gambiae* S sequencing project is in collaboration with MalariaGEN headed by Dominic Kwiatkowski of Oxford University and The Wellcome Trust Sanger Institute and our *A. gambiae* M sequencing project is in collaboration with InfraVec headed by Andrea Crisanti of Imperial College **(7D3)**. In each project, we followed an experimental design similar to that described in the previous reporting periods in which large numbers of females were infected with gametocytes and then scored for their susceptibility. Improvements to this design include that these infections were carried out in the field with blood from local gametocyte carriers rather than with the NF54 lab parasite. Additionally, the colonies used were newly created and have abundant genetic diversity **(7D1)**. Below, we present the figures for the larger scale S infection in which 435 individuals were fed on blood from a single carrier. The phenotypic distribution **(Figure 7.2**, top**)** shows that the individuals chosen for sequencing are clear outliers.

For each of 58 individuals that fell into phenotypic extremes, we sequenced to ~30x depth. This has resulted in an enormous amount of genetic diversity sampled from this colony. We have filtered down from more than 22 million SNPs identified to a set of 6 million which are at reasonable frequencies in the population and which we have now carried out tests on to examine association with infection intensity. We have used PLINK (Purcell et al 2008) logistic regression and adaptive permutations to assess significance. Highlighted in **Figure 7.2** (bottom) are the

genomic regions showing the strongest peaks of association. We are now in the final stages of characterization by knocking down candidate genes in these highly significant regions and determining the impact on infection intensity. We aim to submit two manuscripts by the end of the year on these two sequencing projects **(7D3)**.

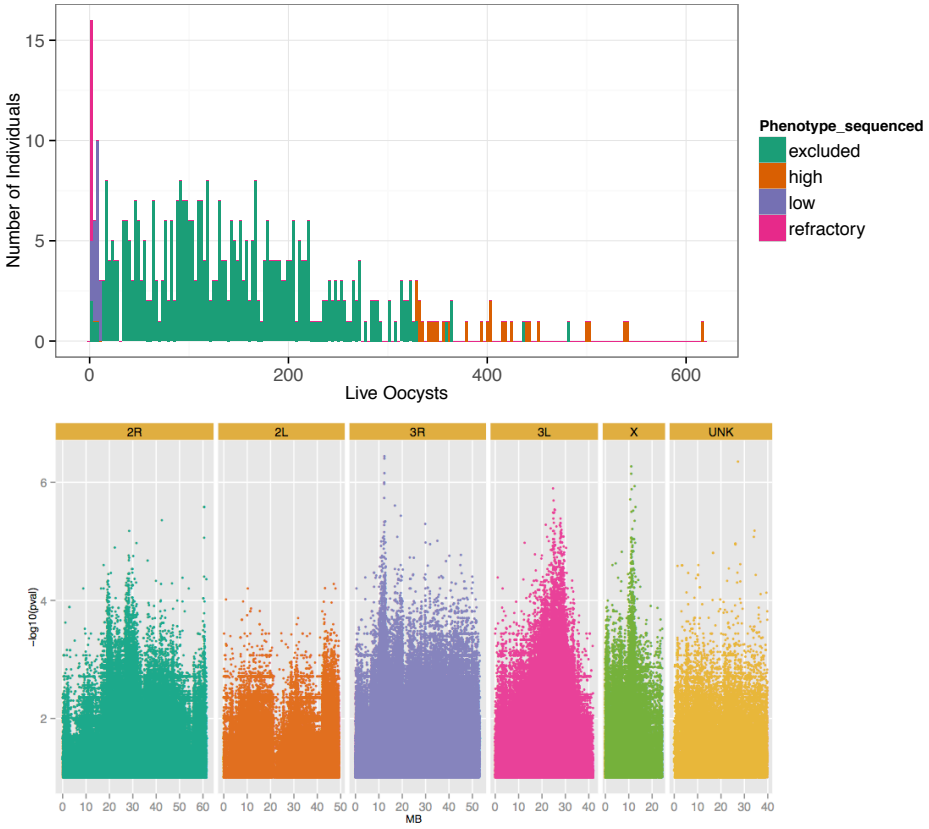

Fig WP7.2. Phenotypic distribution of infection intensity from the set of individuals chosen for genome sequencing (top). Manhattan plot showing genomic locations of SNPs and genomic regions showing significant association with infection intensity (bottom).

Additionally, we exploited the fact that mosquito gut bacteria can influence the outcome of *Plasmodium* infections and at the same time exert a strong drive on genetic variation through natural selection to identify genomic regions that affect gut bacteria impacting on malaria infections. For this reason, we used our SNP chip genotyping platform, microarray expression profiling, RNAi-mediated silencing and 454 pyrosequencing of the microbiome (Stathopoulos et al., in production) (**7D3**). We identified 138 *An. gambiae* genes to be genetically associated with the outcome of the enterobacterium *Serratia marcescens*. Silencing of three genes encoding type III fibronectin domain proteins (*FN3Ds*) increased the *Serratia* load and altered the gut microbiota composition in favor of *Enterobacteriaceae*. These data suggest that natural genetic variation in immune-related genes can shape the bacterial population structure of the mosquito gut with high specificity. Additionally, silencing the gene encoding the gustatory receptor Gr9 that is also associated with the *Serratia* infection phenotype drastically increased *Serratia* levels suggesting a behavioral immune response following *Serratia* infection. Our findings reveal that the mosquito response to oral *Serratia* infection comprises both an epithelial and a behavioral immune component. We are now investigating the impact these interactions on Plasmodium infections.

Another Gram-negative bacterium we tested using this platform is acetic acid bacterium of the genus Asaia that is stably associated with many mosquito laboratory and field populations (Mitraka et al., 2013). While the results of SNP chip and whole genome sequencing analysis are in progress, we observed a dramatic increase in the *A. gambiae* developmental rate when additional bacteria are introduced into the the larval stage through the rearing water which leads us to the conclusion that Asaia plays a yet undetermined crucial role during the larval stages.

O5. A Popgen database has being developed that handles the population biology data. This database is part of VectorBase, a genomics resource for invertebrate vectors of disease (www.vectorbase.org). At the beginning of the project we identified a lack of a structured system for storing population data: sampling sites, phenotypes, etc. Along with collaborators at Washington State, Cornell, NESCent and others from the GMOD project we have designed a 'Natural Diversity' extension to GMOD. This database is capable of storing large-scale population genomics and phenotyping data and is fully interoperable with the rest of the Chado/GMOD system. Since Popgen had to be based on the Chado schema that drives VectorBase, we had to expand the ontologies used in order for them to cover the corresponding domains. Terms that could not be described by already established ontologies but needed to be included, were added to VBCV, a newly built controlled vocabulary meant for our internal use only, giving us the possibility to add yet uncategorized data into our database. The incorporation of IRBase into PopGen led to an additional venue of data submission. We allowed for the usage of the free open source ISA-Tab application suite, to enter the data into spreadsheet-like forms, which we developed. The use of ISA-Tab greatly assists with formatting, validating and selecting ontology terms, processes that were not possible using simple spreadsheets. Finally, to record phenotype data we adopted the Phenote application that also makes it possible to format the data according to the template and also to utilize ontologies. In addition, we developed a design for the generation of ontology domain that would describe and accommodate storage, presentation and meta-analysis of TBD and TBR data obtained by the project. This domain will be constructed as part and incorporated into IDOMAL, the malaria ontology, which would enable its efficient usage in its natural context, allowing for maximized interoperability between related databases. We improved the IDOMAL's structure and edited some inaccuracies, and importantly have introduced a TransMalariaBloc specific section on natural products and remedies. A production version of the Population Biology database was delivered on the VectorBase website to the community **(2D2&7D4)**. The database was demonstrated and tutorials with end users were performed at the Kolymbari vector meeting held in July 2013 in Crete (MacCallum, Kolymbari Popbio). We also continued to develop ontologies that would help us archive and retrieve the data from the Popbio database (Topalis et al., 2013).

WP7: Fitness Costs and Benefits of Resistance

WP #:	8	**Months:**						1-54
WP title:	Fitness Costs and Benefits of Resistance							
Activity type:	RTD							
Participant #:	1	2	3	4	5	6	7	**Total**
Person months/participant:	0	0	0	0	47	84	0	**131**

Summary: In order to assess the vectorial competence and cost of infection in several mosquito populations, colonies of *A. gambiae* M, *A. gambiae* S and *A. arabiensis* were established. Sympatric and allopatric infections (infections of mosquitoes by their local parasites or by parasites from other African regions) revealed that sympatric interactions result in lower infection intensity suggesting local adaptation.

A negative control of infection by heat inactivation of gametocytes was tested and validated as heating non infected blood did not affect the mosquito fitness. Infected and non infected *A. gambiae* M were compared for their life history traits and did not reveal any infection cost in standard laboratory conditions at preliminary analysis.

Moreover, in the current reporting period, analysis of the data showed a limited infection cost depending on parasite isolates in standard laboratory conditions. In conditions of limited nutritional resources, a cost of infection (exposure to parasite) was observed for all parasite isolates. This is the first clear evidence of the cost of infection for the vector in the natural system *A. gambiae-P. falciparum*.

We examined the resistance of mosquitoes and parasites to seasonally variable, and occasionally

extreme, weather conditions and showed that *A. gambiae* can survive extreme temperatures and low humidity, while *P. falciparum* can complete sporogony at temperatures above what was described previously. We also showed that exposure to the insecticides DDT or bendiocarb significantly reduced the prevalence of infection in mosquitoes carrying resistance alleles for each of these insecticides, respectively, which in turn increases the cost of *P. falciparum* infection. Finally we showed that *A. gambiae* (S form), *A. coluzzii* (M form) and *A. arabiensis* have similar competence for different haplotypes of parasites and therefore that genotype*genotype interactions are more likely to apply at the individual level than at vector species level.

Objectives: WP8 aimed to initiate studies of vectorial competence in *A. gambiae* and *A. arabiensis* colonies representative of wild populations, as well as transgenic mosquitoes generated by WP5, and examine the fitness cost to these mosquitoes when infected with field isolates of *P. falciparum*. These aims were broken down into 4 objectives:

O1. Determine the vectorial competence and infection/resistance costs using different mosquito populations infected with sympatric and allopatric parasites;

O2. Determine the environmental effect on vectorial capacity and fitness following infection;

O3. Determine the mosquito genetic component on vectorial capacity and fitness following infection; and

O4. Determine the vectorial capacity and fitness of transgenic mosquitoes.

Modifications: O4 was not implemented and thus M2 was not obtained, as transgenic lines were not available within the timeframe of the project.

O1. Experimental infections were carried out using field parasite isolates from Burkina Faso to challenge *A. gambiae* M from Burkina Faso and Cameroon. These experiments revealed higher parasite load in allopatric combinations than in sympatric but the prevalence is not dependant on sympatric/allopatric combinations. These results suggest adaptive evolution between parasites and mosquitoes to minimize infection intensities but yet allow transmission (Harris et al., 2012). We also initiated a study of comparative susceptibility of sympatric *An. gambiae* M, S and *An. arabiensis* using colonies of local mosquito populations from Burkina Faso **(8M1)** infected field parasite isolates. For each infection, the 3 colonies were raised in a synchronized manner and females were exposed to the same infectious blood. Mosquito guts were dissected 7 days later to measure the level of infection. The size of the mosquito and the size of the blood meal were measured in order to weight the level of infection with these parameters. Individual oocysts were isolated and genotyped for seven microsatellites to determine if the 3 vector species transmit the same genotypes when exposed to the same pool of parasite haplotypes.

The fitness cost of infection was assessed in *A. gambiae* M from Burkina Faso exposing female mosquitoes to sympatric field parasite isolates. Control mosquitoes were fed on the same blood samples following heat inactivation. Fed mosquitoes were then kept individually in standard laboratory conditions to measure their life history traits. The model revealed limited effect of infection on survival but interestingly, an interaction between the blood donor (parasite isolate) and the infection status (infected/ exposed but not infected/ non-exposed) was observed, suggesting that the effect of infection depends on the parasite isolate. No effect of infection was observed on fecundity.

To compare fitness of infected vs non-infected mosquitoes we considered the possibility of using the same blood treated to become non infectious as negative control. Empiric experiments showed in the lab that gametocyte containing blood heated at 42° for 15 min always become non-infectious. We therefore tested the potential effect of heating blood on mosquito fitness. We used non infected blood that was heated to simulate a gametocyte inactivation and measured life history traits in mosquitoes fed on heated or non heated blood. The analyses of 5 replicates (485 mosquitoes in total) showed no effect of blood heating on mosquito survival and fecundity validating the use of heated blood as negative control of infection for fitness studies.

O2 (8D1). The environmental effect on vector competence and the fitness cost of infection were further investigated. The seasonal climatic conditions with daily variations were recorded in the field and mimicked in climatic chambers for the 3 seasons (August: high humidity and medium

temperature; December: low humidity and lowest temperature and February: low humidity and high temperature). Mosquitoes maintained under December conditions survived longer than the mosquitoes maintained under August and February conditions, which showed similar survival rates. This highlighted that *A. gambiae* can survive extreme temperatures and low humidity when it varies along the day. Similar finding were done for parasites, as *P. falciparum* was able to complete the sporogony at temperatures above what was described so far (with T° peaks at 36°C). The seasonal conditions affected the level of infection. Current work will now analyze the dynamics of sporozoites reaching the salivary glands to measure both vectorial capacity and vector competence. Fitness cost of infection was not affected by seasonal variation. The stress conditions identified during the preceding reporting period (sugar resource) therefore appears to be more important for infection fitness cost than the potential stress caused by seasonal conditions.

We also identified the presence of insecticide as a potential environmental stress that may influence vector competence and ultimately infection cost. Insecticide resistant mosquito strains were exposed to insecticides for 18h before infection with *P. falciparum*. Exposure to insecticides DDT or bendiocarb significantly reduced the prevalence of infection in mosquitoes carrying the Kdr or Ace1 resistance alleles, respectively. Moreover, exposure to insecticide increases the cost of *P. falciparum* infection. Field caught mosquitoes selected for resistance to DDT died before non-infected control mosquitoes. Taken together, these results show that the presence of insecticide resistant mosquitoes through the use of insecticides does not increase the vectorial competence or capacity of resistant mosquitoes. However, as discussed in O3, other interactions between insecticide resistance and infection may have the opposite effect, increasing malaria transmission.

O3 (8D1). We investigated the genetic component of vector competence. We previously described parallel experimental infections of *A. gambiae* M and S, and *An arabiensis*. Subsequently, we analyzed the genetic composition of the parasites that developed in the 3 species. Microsatellites analysis revealed similar genetic composition of oocysts in the 3 species for 3 different parasite isolates. This suggest that the 3 species have similar competence for different haplotypes of parasites and therefore that genotype x genotype interactions are more likely to apply at the individual level than at vector species level.

We also investigated the effect of insecticide resistance alleles on vector competence. We used mosquito colonies that share the same genetic background (kisumu), introgressed either for the Kdr or the Ace1 resistance gene. Here the effect of insecticide resistance was tested in absence of insecticides and revealed higher prevalence of infection in insecticide resistance mosquitoes. These results therefore suggest an adverse effect of insecticide resistance on malaria control as compared to insecticide susceptible mosquitoes.

During this reporting period three research articles reporting the deliverable **8D1** were published (Alout et al., 2013; Mollahosseini et al., 2012; Sangare et al., 2013). A review article highlighting how environmental factors influence vector competence, also associated with **8D1**, was also published (Lefevre et al., 2013). Additional manuscripts are in preparation reporting data generated by this WP.

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d) Description of potential impact, dissemination activities and exploitation of results

Malaria affects almost half of the world population and kills about 1 million people every year, while its socioeconomic impacts especially on endemic countries are devastating. Whilst the development of new therapeutics for symptomatic treatment of patients is imperative, this alone cannot lead to elimination of malaria, which is now a top priority in the global malaria agenda. Elimination of the disease can be achieved by either of three ways or indeed a combination of them: vector management and control, a vaccine-mediated prevention of new infections, and blockade of transmission from infected to otherwise healthy people. TransMalariaBloc has aimed to make a significant impact on achieving the latter through informed development and detailed assessment of transmission-blocking vaccines, drugs, and remedies or through generating mosquitoes that do not transmit the disease. At the end of this first phase of the project, we can report. At the end of this initial phase of the project, we can confidently report that transmission blocking can work and that there is substantial groundwork and a critical mass to take these studies further toward exploitation. Our conclusion and vision from analysing the data we, and others prior to this project, have produced over the years and by reviewing past breakthroughs in the elimination of infectious diseases is that transmission blocking may be a path to success. Specifically:

- TransMalariaBloc has been one of the cornerstones of the transmission blocking concept that has been now established as an important framework in the fight against malaria. Therefore, TransMalariaBloc has had substantial impact on its field.
- TransMalariaBloc has contributed or directly given rise to many new consortia and projects supported by the Medicines for Malaria Ventures (MMV) on transmission blocking drugs, the Malaria Vaccine Initiative (MVI) on the impact of transmission blocking interventions in field settings, the Bill and Melinda Gates Foundation on the impact of transmission blocking interventions on the population level and others. Therefore, our project has had substantial impact in initiating new thrusts aiming at exploitation of the results and development of interventions.
- TransMalariaBloc has generated results that could be directly or indirectly exploited towards transmission blocking. This includes transmission blocking vaccines, transmission blocking drugs and transmission blocking remedies. Two well-understood transmission blocking vaccine candidates have been shown to be the most promising: P25 and P230. The former is already in Phase I trial. Robust assays have been developed to report for transmission blocking drug efficacy, and indeed hundreds of such drugs have been discovered through spin-off projects in collaboration with non-academic partnerships described above. One of these drugs is targeting a pathway that is essential for mosquitoes to survive after a bloodmeal. This drug is currently being tested in field settings. Plant extracts with transmission blocking capacities have been characterized, which now need to be further studies towards the development of novel interventions. Indeed, these data have precipitated the formation of a new enterprise (http://www.proherbalcare.com/) that would assist in further exploitation of these data.
- TransMalariaBloc has generated experimental protocols and facilities that can be used in future malaria transmission blocking research. The protocol we established for mosquito infections with malaria parasites directly sampled from infected children and the coupling of this protocol to the various transmission blocking interventions we have developed is a significant breakthrough in linking laboratory discoveries to field tests. Coupled with a state-of-the-art facility we generated to carry out such work in Bobo Dioulasso, Burkina Faso, this protocol can be used for future exploitation of our results and discoveries made by others. The malaria house facility we contributed to establish in Iganga, Uganda, to test anti-malarial vaccines could be used for Phase I trials of transmission blocking vaccines.
- TransMalariaBloc has educated, trained and contributed to career development of several new investigators that will continue the research in field of malaria transmission. Many of these investigators have now established their own research programs continuing the

translational work of data derived from the project in collaboration with academic and non-academic sponsors.

- TransMalariaBloc has identified several new transmission blocking targets and has developed new screening assays that can fuel future translational and applied research for many years. A new consortium aiming to explore the value of new parasite targets for the development of transmission blocking vaccines has been already formed with the inclusion of additional partners specializing on anti-malarial vaccines. With regards to mosquito molecules that can be used as transmission blocking targets, a new concept has emerged from discoveries made in the context of the project, whereby antibodies ingested by with the blood would render mosquitoes incapable of controlling opportunistic infections and therefore die before transmitting the disease. We are now validating this concept in field settings and are seeking funds to further extent the project.
- TransMalariaBloc has revealed that antibiotics present in human blood increase the capacity of mosquitoes to transmit malaria. Since antibiotic use in Africa is high and recommended in WHO-led disease elimination programmes, these data are of particular importance to public health. Towards exploitation of these data we now aim to directly test antibiotic treatment of malaria-infected patients and examine a number of antibiotics that are currently in use in malaria-endemic countries. Following these work, specific recommendations can be made to national and international health services for tightly regulated antibiotic prescription in areas with high malaria transmission, prescription of specific antibiotics to malaria-infected patients, and couple mass antibiotic administration programs to increased bednet coverage.

e) Public website address

http://www.transmalariabloc.net