ENAROMaTIC Project

FINAL REPORT

January 29, 2013

1. Executive Summary

Malaria, the deadliest of the known insect-born diseases, is transmitted to humans by Anopheline mosquitoes. The goal of ENAROMaTIC has been to discover compounds of natural or synthetic origin capable of interfering with the olfactory function of mosquitoes and their capacity to detect the presence of humans in their environment and transmit malaria parasites through blood meals.

Toward this objective, the consortium has undertaken a series of studies and has achieved the main objectives listed in the research plan as follows.

1. Several odorant binding proteins (OBPs) and odorant receptors (ORs) of *Anopheles gambiae*, which participate in the process of human host odor recognition, have been cloned, expressed in bacteria and/or insect cells and used in screening and crystallization studies.
2. Protein (for OBPs) and cell-based (for ORs) screening assays allowing fast analyses of synthetic and natural (aromatic plant-derived) compound collections for the presence of olfaction-based behavior modifiers have been established and relevant screens were carried out.
3. To enhance the effectiveness of the screening process, specific reagents and methods were developed, which allowed the establishment of co-expression patterns for a number of the selected OBPs and ORs in female antennal sensilla. Because co-expression of OBPs and ORs recognizing common ligands is prerequisite for efficient odor recognition, co-expressed proteins received priority in the screening efforts.
4. The capacity of essential oils (EOs), selected by OBP *in vitro* screens, to trigger changes in female *A. gambiae* behaviour was assessed by *in vivo* behavioural and electrophysiological assays and a total of 35 EOs were found to have significant repellent activities. Nine EO constituent compounds and derivatives having strong repellent properties were also identified. The precise repellent effects of the five most active compounds and binary and ternary mixtures thereof have been established.
5. The crystallization of several OBPs and structure determination of five ligand-bound OBPs served as the basis for modelling studies, which resulted in predictions for compounds, including derivatives of the identified repellents, with better OBP binding potential. Of twenty such synthetic compounds that were assessed for binding to specific OBPs and *in vivo* functionality, five were found to have strong repellent activities.
6. The four most active natural repellent compounds identified by *in vivo* assessments and mixtures thereof have been evaluated for acute neurotoxicity and chronic cytotoxicity effects on rodent cortex and midbrain cultures grown on the surface of microelectrode array chips. The acute toxicity assays suggested that the newly discovered mosquito repellents should not to be toxic unless ingested in considerable quantities. The chronic neurotoxicity assays suggested that direct administration of the two most active repellent compounds to the cultures produces changes in midbrain network activity similar to those of DEET, whereas administration of the other two causes no impairment of midbrain network activity.
7. Efficacy assessments of the same four most active natural repellent compounds, carried out initially in model huts in Nigeria, have shown one of these compounds to display repellency equivalent to DEET, while specific binary mixtures of the new compounds produced significantly higher repellent effects. The testing of the same compounds in rooms of family-occupied dwellings in a selected village in Nigeria produced results essentially identical to those obtained from the model huts.
8. Procedures for the protection of intellectual property associated with the discovery of the new natural repellent compounds are reaching completion and discussions with relevant companies that expressed an interest in their commercial exploitation are in progress.

Overall, these results have demonstrated both the feasibility of isolating new, safe-to-use olfaction-based mosquito behavior modifiers from natural sources and the validity of the principle that rational design of novel compounds with enhanced bioactivities based on modeling studies that capitalize on known OBP three dimensional structures is feasible. Thus, it is felt that the ENAROMaTIC consortium has achieved fully its mission for a contribution toward the reduction in malaria transmission to humans by targeting the olfactory capacity of the mosquito disease vector.

For further information regarding ENAROMaTIC please visit www.enaromatic.org.

2. Summary description of project context and main objectives

Blood-feeding insects carry many potentially lethal diseases, including encephalitis, malaria, and yellow fever. The most deadly of the insect-born diseases is malaria. It is caused by the parasite *Plasmodium falciparum, which is* transmitted to humans by female anopheline mosquitoes, particularly *Anopheles gambiae*, during blood feeding. The most recent estimates for human losses to malaria currently stand at over 400 million of new clinical cases and approximately 1 million deaths each year, the latter involving particularly children under the age of five.

To date malaria control has relied mainly on repressing the insect vector–human target interaction. Insecticides have helped to contain malaria in the past and even now, commercially available products to control mosquitoes are mostly insecticides and insecticide-treated bed nets. However, because insecticides proved to have severe adverse effects on public health and the general ecosystem, as well as being ineffective after prolonged use due to the rise of insecticide resistance in the targeted vector populations, new methods for controlling the disease vector are needed.

The means that could potentially be employed to control the sizes of mosquito disease vector populations and frequency of contact between vectors and human hosts rely on three distinct areas of research, (i) development of new effective but environmentally safe insecticides; (ii) development of genetically modified mosquitoes that maintain unreduced survival vigor in natural environments but are refractive to parasite propagation and survival; and (iii) discovery of new compounds, mostly volatiles, for use as agents disrupting the normal olfactory capacity of female mosquitoes thus preventing them from identifying their human hosts, obtaining blood meals from them, and transmitting the malaria parasite in the process. ENAROMaTIC's effort has focused on the third of these areas of discovery.

ENAROMaTIC has investigated the mechanisms providing the specificity of odor recognition with emphasis on the initial stages of this process in anopheline mosquitoes. Besides the acquisition of important new knowledge on this important physiological process, the ultimate goal of the project has been the identification of novel compounds capable of interfering with the mosquito's capacity to recognize odors of human origin and transmit the malaria parasite to humans through blood feeding.

Until the onset of the ENAROMaTIC project, most efforts to understand anopheline mosquito olfaction had focused on either of the two major groups of proteins involved in odor recognition, odorant binding proteins (OBPs) and odorant receptors (ORs), which are usually studied separately. In contrast, because it was clear that the olfactory process requires the coordinated action of both of these components for efficient perception of odor stimuli, ENAROMaTIC's strategy has been to tackle the target of interfering with the mosquito olfactory function by manipulating concurrently both components of this system. Thus, at the heart of ENAROMaTIC research laid the fundamental principle that optimal odor recognition tuning and sensitivity are dependent on the availability of odor-matched OBP-OR pair combinations within single olfactory sensilla.

It has been anticipated that successful development of the proposed studies would advance significantly the understanding of the mechanisms that control odor recognition in mosquitoes. Most importantly, it was felt that the identification of multiple disruptors of host seeking behavior of female mosquitoes would provide new and effective tools to be employed in the effort to reduce the incidence of contact between the human host and the mosquito disease vector and produce a commensurate reduction in the spread of malaria transmission. Because a natural pathway essential for reproduction was targeted, the probability of occurrence of resistance at the level of OBPs or ORs is minimal because of the impact of the relevant mutations on the organism's capacity to perceive and respond to *bona fide* host odors. Furthermore, the availability of multiple high affinity OR ligands, would permit their sequential use for short periods of time, thus further minimizing the (already minimal) risk of resistance development in the mosquito populations due to prolonged exposure to single control agents.

To achieve ENAROMaTIC's objectives, a significant number of prerequisites had to be fulfilled including the availability of

- *rationally selected OBP and OR targets that likely participate in the host seeking process;*
- *collections of synthetic or natural compounds to be examined with appropriate screening methods for the presence of desired functional characteristics;*

- *fast in vitro screening methods for reliable initial analyses of large collections of compounds for the purpose of defining subsets displaying physiologically relevant binding capacity for specific OBP and OR targets and specific effects on the function of cognate ORs;*
- *efficient methods for precise evaluation of the consequences of application of the selected subset of compounds on mosquito behavior in vivo;*
- *methods for analyzing important structural features of OBP ligand binding pockets and determining ligand/OBP structure-function relationships;*
- *capacity for reliable modeling of ligand derivatives with enhanced binding affinity and function;*
- *capacity to synthesize the ligands and modeled ligand derivatives in native and radio-labeled forms needed for the purpose of evaluating their binding and functional properties.*

Additionally, prior to establishing unequivocally their usefulness as agents that may interfere with the female mosquito's capacity to identify its human host, the compounds selected as the strongest candidate modifiers of olfaction-based female mosquito behavior should be evaluated, at least at an initial level, for

- *lack of cytotoxicity;*
- *efficacy under field conditions.*

Accordingly, the following broad scientific objectives were defined:

1. Cloning and expression of OBPs and ORs judged to play important roles in the process of human host odor recognition by the female mosquito, the vector of the disease;
2. Discovery of disruptors of the host seeking behavior using a number of novel as well as previously developed *in vitro* high-throughput screening (HTS) systems in conjunction with collections of synthetic compounds and natural compound mixtures derived from aromatic plants, which are rich in volatiles;
3. Identification of OBP-OR combinations recognizing common ligands and potential disruptors of host seeking behavior and determination of their distribution in the female mosquito antennal sensilla;
4. Determination of *in vivo* functional properties of newly identified lead compounds by electro-physiological and behavioral assays involving female (and male) mosquitoes;
5. Crystallization and crystal-based structure determination of a number of model OBPs selected on structure and functionality-related criteria, modeling of ligand fitting into ligand-binding pockets and design of derivatives having better binding properties;
6. Synthesis of ligand derivatives and validation of improved binding to cognate OBPs and enhanced functionality in the *in vivo* electrophysiological and behavioral asssays;
7. Evaluation of lead compounds deemed to be effective with regard to disruption of normal female mosquito behavior for potential cytotoxicity on mammalian cells using model mammalian neuronal network biosensors;
8. Testing of the most promising disruptors of olfaction-based mosquito behavior at sites in an African country where malaria is endemic to determine efficacy under conditions that simulate the sites of eventual application of newly developed products.

These objectives would be fulfilled through the realization of 5 interconnected working Sections each encompassing specific work packages and tasks therein, as summarized below.

Section 1 objectives: The objectives of this section were to develop and employ high throughput *in vitro* screening assays for identification of OBP- and OR-specific ligands; define ligand-matched OBP-OR pairs; and employ additional *in vitro* tests to assess the functional relevance of ligand-matched OBP-OR pair combinations in the olfactory process. Different groups of natural or synthetic chemical molecules or mixtures thereof were to be screened against the defined OBP and OR targets for ligand lead activities. These objectives were pursued in the context of the 3 work packages, which are briefly described below.

WP1: identification of specific ligands for 10 selected OBPs shown to have a strong expression bias toward female mosquito antennae. Tasks under WP1 included the

- *expression and purification of recombinant OBPs (**Task 1.1**);*
- *screening for ligands for specific OBPs amongst a number of chemicals known to elicit electrophysiological responses in female mosquito antennae and derivatives thereof **(Task 1.2**);*
- *screening of extracts (essential oils) derived from available collection of plants and known to contain OBP-specific binding activities (**Task 1.3**).*

WP2: identification of ligands with specific agonist and antagonist activities 11 selected ORs. The targets included 9 ORs displaying a pronounced bias for expression in female antennae, OR1, which has a known ligand and OR7 (Orco), the heteromerization partner of all ORs. Tasks under WP2 included the

- *generation of transformed cell lines expressing stably the selected ORs and relevant Ga subunits of trimeric G protein complexes (**Task 2.1**);*
- *cell-based high throughput screening for the presence of OR ligands amongst (i) chemical compounds known to trigger odorant-related responses in mosquitoes and (ii) other chemicals shown to possess specific OBP binding activities in WP1 (**Task 2.2**);*
- *screening for the presence of OR ligands amongst the members of a combinatorial chemical library shown to possess OBP ligand activity (**Task 2.3**);*
- *preparation of extracts from available endemic plant collection (**Task 2.4**);*
- *screening of plant extracts for the presence of OR ligands (**Task 2.5**).*

WP3: confirmation of functional relevance of defined ligand matched OBP-OR pairs using in vitro methodologies. This confirmation was important because it is the prelude to the more detailed studies required for the development of novel ligands capable of interfering effectively with the mosquito's olfactory competence. Tasks under WP3 will included the

- *assessment of enhancement of in vitro ligand-dependent OR activity by ligand-matched OBPs (**Task 3.1**);*
- *generation of anti-OBP and anti-OR antibodies (**Task 3.2**);*
- *examination of co-expression patterns of ligand-matched OBP-OR pairs in female olfactory sensilla (**Task 3.3**).*

Section 2 objectives: The objectives of this section have been to (i) crystallize and obtain crystal structures for selected OBPs; (ii) model interactions of ligands in ligand binding pockets; (iii) design and synthesize ligand derivatives with enhanced potential; and (iv) assess functional properties of ligand derivatives *in vitro* and *in vivo*. These objectives were pursued in the context of the 3 workpackages, which are briefly described below.

WP4: crystallization and structure determination of OBPs of known ligand binding specificities. Tasks under WP4 included the

- *production and purification of recombinant OBPs (**Task 4.1**);*
- *crystallization of recombinant proteins (**Task 4.2**);*
- *structure determination of crystallized proteins (**Task 4.3**).*

WP5: modelling of known and newly discovered ligands into cognate OBP ligand binding pockets and designing of ligand mimetics predicted to have improved properties. Tasks under WP5 included the

- *modeling of OBP-ligand interactions and predictive design of ligands with enhanced binding characteristics (**Task 5.1**);*
- *site-directed mutagenesis to confirm QSAR predictions arising from modeling studies (**Task 5.2**).*

WP6: synthesis of native and radiolabeled ligand derivatives and assessment of their in vitro binding characteristics and functional properties. Tasks under WP6 included the

- *synthesis of radioactive ligands and ligand derivatives predicted to have increased binding affinities (**Task 6.1**);*
- *binding assays using wild type and mutant OBPs and radioactive and non-radioactice ligand derivatives (**Task 6.2**);*
- *in vitro functional assays using ligand derivatives with desirable binding properties (**Task 6.3**)*

Section 3 objectives: The objectives of this section were to (i) validate the functionality of identified ligands and cognate OBPs and ORs determined through the work carried out under WPs 1-6 using *in vivo* electrophysiological and behavioral assays; and (ii) classify the identified ligands in terms of *in vivo* effects on female mosquitoes. A final objective of this Section was to examine the *in vivo* effects of selective OBP expression in the antennae of male and female mosquitoes and correlate the reduction in specific OBP expression to physiological responses against administration of the relevant ligands. These experiments would not only establish unequivocally the role of specific OBPs in the process of host recognition but also deduce the extent of functional redundancy existing in the mosquito olfactory system. These objectives were pursued in the context of a single workpackage, which is briefly described below.

WP7: testing the biological activity of all identified ligands and cognate OBPs and ORs using in vivo electrophysiological and behavioral assays. Tasks under WP7 included

- *electrophysiological recordings (**Task 7.1**);*
- *behavioral assays (**Task 7.2**);*
- *specific OBP and OR RNAi injections and functional responses of mosquitoes expressing reduced levels of cognate OBPs to ligand administration (**Task 7.3**).*

Section 4 Objectives: The objective of this section was to undertake a preliminary safety evaluation of newly discovered olfactory disruptors for lack of cytotoxicity effects through the use of neuronal network biosensors. These objectives were pursued in the context of a single workpackage, which is briefly described below.

WP8: employment of mammalian neuronal network microelectrode array (MEA) neurochips for establishing the lack of cytotoxicity of selected ligands for mammalian cells. Tasks under WP8 included the

- *culture of rodent cortex and spinal cord on the surface of microelectrode array neurochips (**Task 8.1**);*
- *analysis of the electrical activity patterns obtained from neuronal networks grown on MEA neurochips after application of ligand leads. (**Task 8.2**).*

Section 5 Objectives: The objectives of this section were to assess the efficacy of a small number of olfactory disruptors, selected on the basis of the *in vivo* experimentation results of WP7 and the preliminary safety assessments of WP8, at model field sites in Africa. These objectives were pursued in the context of a single workpackage, which is briefly described below.

WP9: application of selected ligand mimetics under field conditions in an African country (Nigeria) where malaria is endemic and assessment of field efficacies. Tasks under WP7 included the

- *protocol development for experimental hut studies and A. gambiae population structure survey in experimental test sites (**Task 9.1**);*
- *analysis of effects of olfactory disruptors on host seeking behavior of A. gambiae under experimental hut conditions (**Task 9.2**);*
- *assessment of efficacy of olfactory disruptors on host seeking behavior of A. gambiae under field conditions (**Task 9.3**).*

3. Description of the main S & T results/foregrounds

The progress that has been achieved toward the realization of the 8 project objectives described in the previous section may be summarized as follows:

1. ***Cloning and expression of OBPs and ORs***

For this objective, a total of 12 OBPs and 11 ORs selected as the basis of the ENAROMaTIC studies based on previously determined expression profiles in male and female antennae and demonstration that they are expressed predominantly in female olfactory sensilla, have been cloned in appropriate expression vectors and expressed in bacteria (OBPs) and lepidopteran insect cells (OBPs and ORs).

Recombinant OBPs expressed in bacteria were purified after being subjected to a denaturation-renaturation treatment and their structure and purity determined by circular dichroism (CD) and gas chromatography-mass spectroscopy (GC-MS) analyses. OBP expression in insect cells resulted in secretion of native polypeptides in the media of the expressing cells and purification from the latter using affinity purification protocols for appropriate tags added to their termini. Subsequent studies (summarized below) have demonstrated that the isolated OBPs were also functional in terms of ligand binding properties.

For the case of the ORs, successful functional expression in insect cells has been achieved as documented by a number of criteria, which included (i) biochemical assays (for quantitative assessments) and immunolocalization tests (for confirmation of cellular plasma membrane localization) involving custom made antibodies against native proteins (for two ORs), antibodies against specific epitope tags attached to the recombinant OR termini (for all ORs); and (ii) functional assays (for establishment of functionality) using either known specific ligands [for 4 deorphanized ORs co-expressed with OR7 (Orco)] or a generic, non-specific ligand activating all receptors (for 6 orphan ORs co-expressed with Orco).

2. ***Discovery of ligands that may disrupt mosquito host seeking behavior***

For this target objective, protein-based HTS assays based on fluorescent reporter molecules were established for the cloned OBPs. These assays allowed undertaking of screening tests in microtiter plates with readouts being read in automated plate readers.

Subsets of the purified OBPs have been employed for the screening of (i) a large combinatorial library of chemical compounds of known structures available to one of our partners; (ii) an additional collection of 120 pure chemicals known to be active on mosquitoes, which was available to two of our other partners; (iii) a collection of approximately 200 plant essential oils and fractions thereof containing mixtures of naturally occurring compounds, which have been generated by yet another member of the ENAROMaTIC consortium from various aromatic plants; (iv) pure constituent compounds obtained from OBP-binding essential oils and identified by relevant biochemical fractionation; (v) a small set of chemical compounds with representatives known to have specific bioactivities against specific arthropods including mosquitoes, which were provided to us by one of our collaborating chemical companies; and (vi) another small set of purchased chemical compounds that were predicted to have high binding capacity for specific OBPs (and maybe biological active as well against mosquitoes) based on modeling studies.

The screening tests resulted in the identification of a number of synthetic chemicals as well as multiple plant extracts and fractions thereof containing compounds, which exhibited good binding properties against specific OBPs. They also resulted in the identification of subsets of

the collaborating company compounds that exhibited enhanced binding properties against specific OBPs. Moreover, based on the OBP binding assessments, the binding screens confirmed that modeling studies can be used successfully for predicting compound derivatives having enhanced binding properties against targeted OBPs as compared to the parental compounds. Finally, the OBP binding studies revealed that co-expression of OBPs may lead to OBP-OBP interactions that may extend the odor code of the individual OBPs.

The combined results revealed distinct spectra of binding for each of the examined OBPs and suggested that synthetic compounds and natural ones derived from essential oils may be active on mosquitoes. As described further below, some of these compounds were tested for their biological activity using relevant physiological and behavioral assays and were found to be biologically active against mosquitoes, primarily as powerful repellents.

For the identification of OR ligands, lepidopteran cell lines producing stably the selected ORs and, in some instances, accessory Gα proteins predicted to facilitate and/or maximize functional responses of the ORs upon ligand addition have been generated. These were used as insect cell-based HTS platforms for the screening and identification of (i) aromatic plant extracts containing compounds with ligand-like activities against specific ORs and (ii) new chemical compounds behaving as specific OR ligands. As a possible additional heterologous expression platform for low throughput ligand screening, mammalian cell lines expressing an accessory Gα15 protein were also generated which carried integrated copies of the coding regions of two deorphanized ORs under mammalian promoter control in their genomes.

Prior to actual compound screening, a number of different reporter systems were tested in conjunction with two of the generated cell lines expressing stably ORs in order to identify the reporter system providing the most robust and reproducible functional responses in the transformed cell lines. The different reporter systems tested included (i) Ca^{2+}-fluorescent reporter probes (Fluo3 and Fluo4, evaluated by epifluorescence microscopy); (ii) Ca^{2+}-activated luminescent proteins (aequorin and Photina cytoplasmic and mitochondrial versions, evaluated through the use of a microplate reader); and (iii) membrane potential fluorescent dyes (Red and Blue FLIPR dyes, evaluated both by scanning confocal microscopy and through the use of a monochromator microplate reader). Based on sensitivity of responses and ease of use, the system selected for further exploitation was the insect cell-based assay that relied on the expression of a mitochondrially localized photoprotein, which is activated upon Ca^{2+} entry into the cells resulting from the activation of the selected ORs (ligand-gated ion channels), and was amenable to adaptation to HTS formats. For the mammalian cell lines expressing the accessory Gα15 protein, which were considered as an alternative screening system, initial calcium imaging experiments of OR-expressing cells showed that although ORs appeared mediate cell responses to their cognate ligands, their reactivity in calcium imaging experiments was erratic. Moreover, these cell lines proved to be problematic in terms of viability and ease of use. As a result, obtaining reliable results with the mammalian cells has been judged to be of limited value for ligand screening.

Compared to other major systems used for the characterization of insect ORs, which have rather complex and time-consuming assay preparation and handling requirements and require specialized monitoring instrumentation, the newly developed insect cell-based assay, which employs the Ca^{2+}-sensitive photoprotein for receptor response readout, has considerable advantages. Thus, it yields robust and quantitative readouts of physiological responses of *A. gambiae* ORs upon administration of odors and other relevant ligands without requirements for elaborate instrumentation, and is adaptable to use as a medium to high throughput screening platform for fast discovery of OR-specific ligands.

Besides being used for the screening of the synthetic and natural compound collections described above for the identification of specific ligands, agonists and antagonists, capable of acting as modifiers of olfaction-based mosquito behaviors, the insect cell-based screening system afforded the opportunity for discoveries that shed light into previously unknown details of ligand-dependent olfactory receptor function. Specifically, we have documented that a specific class of reagents can be used as a set of generic tools for establishing the functionality of orphan ORs whose ligands have yet to be determined and obtaining enhanced responses from characterised ones without sacrificing specificity. With the use of such generic activation tools, which act on all mosquito OR-Orco heteromers in a non-specific manner, and by modifying appropriately the basic ligand screening protocol we have also been able to identify in our compound collections those ligands acting both as receptor agonists or antagonists for both deorphanized and orphan receptors. Importantly, for compounds proven to act as major mosquito repellents *in vivo*, we were able to establish that these act on many mosquito receptors and deduced the molecular basis for our findings. The latter represents an important discovery because it affords the modeling of new compounds that could have enhanced and longer lasting repellent properties for *Anopheles* as well as other mosquito disease vectors. Therefore, the newly established insect cell-based assay system for mosquito ORs, which employed a genetically-encoded reporter photoprotein, proved to be a convenient, reliable and information-rich tool suitable for screening efforts and pharmacological characterization of mosquito receptors. Among others, this provided specific insights into the molecular basis underlying the function of some of the olfaction-based mosquito behavior modifiers identified through the combination of OBP screens described above, and the physiological and behavioral assays described further below.

3. *Identification and distribution of OBPs and ORs recognizing common ligands*

The aim of this objective has been to evaluate ENAROMaTIC-selected OBPs and ORs with respect to a possible interplay in the recognition of common ligands. Towards this goal their expression and co-localization in mosquito antennal sensilla have been examined and attempts were made to assess a combined functionality of ligand-matched OR/OBP pairs in cell-based activity assay systems. In order to localize OBP and OR mRNAs and proteins specific riboprobes for all selected OBPs and ORs have been synthesized. In addition, several high titer antisera have been generated and verified for specificity. Furthermore, new protocols for separate as well as combined whole mount fluorescence *in situ* hybridization (WM-FISH) and immuno-histochemistry (WM-FISH) have been established.

Through the use of these methods and confocal laser scanning microscopy, the antennal topography of OBP-transcribing support cells and OR-expressing olfactory sensory neurons (OSNs) have been visualized and significant differences in the number and antennal distribution of expressing cells have been detected, with noticeable broad expression of certain OBPs. Both sexes expressed the same ORs and OBPs; however, female antennae generally had considerably higher numbers of expressing cells, representing a possible basis for gender-specific differences in the ability to detect distinct odorants. Immunolocalization studies have confirmed and significantly extended the WM-FISH-results. Specifically, for the first time antisera against *Anopheles* plus-C and classical OBP-types visualized the respective proteins in the sensillum lymph of sensilla trichodea, thus corroborating their function in odorant detection. The co-localization of OBP-types and OBP/OR pairs in antennal sensilla has been examined by extensive two-color double WM-FISH experiments. Through pair-wise testing of all selected OBPs, complete, partial, and non-overlapping OBP expression patterns have been detected and co-expression of multiple OBP-types in single support cells was also established. Moreover, evidence was provided for expression of classical and plus-C OBP48 by different support cells in the same sensillum.

Overall, a complex OBP expression-mosaic was discovered indicating diverse sensilla types with overlapping OBP equipment, thus suggesting cooperation of distinct OBPs with different ORs or the existence of OBP-OBP heteromers with new ligand binding properties. In line with a functional relevance of ligand-matched OR-OBP pairs, double WM-FISH with combinations of OR2-OBP1 and OR2-OBP4 have demonstrated a close association of the expressing cells indicative for sensillar co-localization of the proteins. Finally, unambiguous assignment of OBP-OR co-expression, including determination of the sensillum type housing the proteins, was demonstrated by applying combined WM-FIHC and WM-FISH. Sensillar co-localization was identified for several other OR-OBP combinations, suggesting pairs, interplaying in odorant recognition and functionally testable in forthcoming studies.

Given the development and current availability of the robust and convenient insect cell-based platform for functional assessment of heterologously expressed mosquito ORs, which was described above, the testing of the interplay of ligand-matched OBP-OR pairs is now possible. Due to the late availability of the insect cell–based assay system, however, meaningful results from measurements with OR-expressing cells in the presence of co-localized and ligand-matched OBPs (i.e. OR2-OBP1, OR2-OBP4) have not been obtained yet, but are expected beyond the end of the project.

In summary, the realization of this objective provided a detailed antennal expression analysis of OBP and OR types implicated in host odor recognition and revealed olfactory sensillum co-localization for a number of ligand-matched pairs supposed to play important roles in odor recognition. Together with setting the stage for testing combined functionality of co-localized pairs, the results achieved represent an important step towards the identification and characterization of molecular targets, which may be used in novel mosquito control strategies.

4. *Functional properties of identified lead compounds*

The capacity of extracts (essential oils) obtained from various aromatic plants to effect changes in female mosquito behaviour was examined through the combined use of a specific repellency assay involving *A. gambiae* females and gas chromatography-coupled electroantennograms (GC-EAG) recordings, which were used to identify the chemical nature of the bioactive compounds contained in active extracts. A total of 82 essential oils from different plant families, which were found to contain OBP binding components as described above, were tested using a specific assay, the warm body repellent assay that was developed within the Consortium. These essential oils were tested during the final hours of the scotophase in a randomized design, where each product was tested, in multiple cages, with a total of 250 *A. gambiae* females. Compared to an ethanol control sample, which did not display any mosquito repellent activity, a defined dose of the most widely used mosquito repellent DEET, which was much lower than the quantity normally applied to the human skin for protection against mosquitoes, caused a strong reduction in the number of mosquito landings to the warm body. In addition, 21 of the tested essential oils applied at the same dose to the warm body showed repellence no different from DEET, while a few additional ones showed an attraction trend for mosquitoes. Fractions of the repellent essential oils also showed DEET-like repellence for *A. gambiae* females.

Constituent compounds isolated from 5 strongly repellent essential oils subjected to further analyses were shown to be capable of inducing electroantennogram (EAG) responses from *A. gambiae*. These were identified using specific biochemical fractionation procedures coupled to electrophysiological testing on live mosquitoes [gas chromatography (GC)-linked electroantennogram (EAG) recordings]. Subsequently, their structures were determined through application of relevant methodologies (GC-mass spectrometry) that were available

for this purpose. Through the use of additional behavioural tests involving the physiological (EAG) response-triggering constituents of each essential oil, several behaviour-modifying essential oil constituents and derivatives were identified. These included 3 compounds identified in the essential oils themselves and 5 structurally related compounds that showed DEET-like repellence when tested in the warm body assay at doses similar to DEET. Additional compounds were also identified, which apparently had an opposite type of effect, namely attraction to mosquitoes.

Besides dose responses for the most active repellents in relation to the widely used repellent standard DEET, the effects of binary and ternary mixtures of the active leads at total (cumulative) concentrations equal to those of the individual tested compounds were examined and certain mixtures were found to display repellent activities significantly superior to DEET. Moreover, the most active compound also showed spatial repellent effects against *A. gambiae* females in a wind tunnel that were similar to those of DEET.

Finally, the direct relationship between specific OBP-ligand interactions and physiological responses of the antenna was also demonstrated by RNAi-mediated inhibition experiments, which have shown that elimination of a cognate OBP in RNAi-injected mosquitoes abolishes the antennal electrophysiological responses to the relevant ligand.

The search for additional aromatic plant constituents with strong mosquito repellent activity continues as the majority of the essential oils that were shown to have significant mosquito repellent activities have yet to be subjected to further analyses of the type described above, which would reveal the identity of the constituents that are responsible for the repellent effects. As is also described below, the identified natural products displaying strong mosquito repellent activity are subjected to more extended analyses that involve biochemical studies and modeling of their interactions with various OBPs for the purpose of identifying modifications that may provide additional desirable properties to them. In fact, the same type of in vivo assessment has been carried out for (i) all synthetic compounds modeled by ENAROMaTIC partners based on the structures of compounds displaying strong binding for various OBPs, and (ii) all compounds that were provided by our industrial collaborator and shown to possess both specific OBP binding and ligand activities against specific ORs. As a result of the additional electrophysiological and behavioral assays 9 additional compounds showing DEET-like repellence activities were identified. Besides having added new candidate tools to the arsenal to be used against mosquito disease vectors, these results have also demonstrated the power of modelling studies, particularly when the latter are carried out based on determined structures of both the relevant olfactory proteins and compounds interacting with them (see further below for additional information) and are combined with relevant functional assays capable of confirming or negating the tested hypotheses.

5. *OBP crystal structure-based discovery of novel repellents*

Knowledge of the three-dimensional structure of target proteins provides a starting point for structure-based approaches to repellent/attractant discovery by defining the topographies and electrostatics of the complementary surfaces of ligands and their protein targets. This information can help both the computational and synthetic chemist to optimize compounds by building better interactions with the protein, resulting in improved potency and selectivity. Accordingly, the aim of this broad objective has been to crystallize and determine the three-dimensional structure of female specific OBPs to be used for the structure-based discovery of novel classes of repellents.

Eight classic OBPs and two C-plus OBPs, which contain additional domains, have been expressed in large quantities and purified for crystallization purposes.

High-resolution X-ray data were collected for 6 OBPs (including one, OBP1, whose crystal structure has been previously determined by another group). The five new OBPs included 3 members of the classic group (OBPs 4, 7, 9) and, for the first time, two members of the C-Plus group of OBPs (OBP47 and OBP48). The three-dimensional structures of the crystallized proteins were determined both for unliganded proteins (the apo forms of OBP4 and OBP47) and proteins containing a ligand in their binding pockets (OBP1-DEET, OBP4-1-NPN, OBP4-TC4, OBP4-TC9, OBP4-indole, OBP7-AZO, OBP7-lipid X, OBP9-lipid X and OBP48-PEG). The same studies also demonstrated that under certain conditions OBPs crystallize as dimers that may represent the physiological targets.

Of particular interest was the first high-resolution crystal structure of one of the classic OBPs, OBP1, in complex with the synthetic repellent DEET, which demonstrated experimentally for the first time that OBPs represent valuable molecular targets for repellent structure-based design. In general, the identification of different binding cavities, which varied in shape, position, and solvent accessibility, as well as the nature of the amino acids that form their boundaries provided a better understanding of the differences in the binding specificities of the various OBPs and thus specific hints related to the molecular basis of odorant detection.

Explicit molecular mechanics calculations were also undertaken subsequently in order to examine further (i) the interaction of various potential ligands with OBP1, the single OBP whose structure was known at the beginning of the project, as well as OBPs whose structures were determined by the ENAROMaTIC project; and (ii) predict three-dimensional structures for other OBPs by homology modelling approaches. Thus, protein-ligand interactions were analyzed using both crystal structures (for OBPs 1, 4, 9, 47 and 48) and homology models built using appropriate computing programs (for OBPs 3, 5, 12, 20 and 22). Structural analyses of OBPs highlighted conserved residues comprising ligand-binding pockets. Homodimers and heterodimers were also analyzed with appropriate computational methods and interface residues involved in protein-protein interactions were predicted. To confirm experimentally the validity of the conclusions drawn from these models, the interactions between residues in the binding cavities of selected OBPs and functional groups and surfaces of bound ligands have been confirmed by site-specific mutagenesis. The mutated OBPs were analyzed for their physical properties and ligand binding characteristics and they experimentally validated the predicted functions of the investigated amino acids.

Additionally, comparative studies examining ligand binding to OBP1 and OBP48 homodimers were also performed. The theoretical docking results coincided with the experimentally determined positions of DEET in the OBP1 homodimer, while two ligand molecules were placed in the combined site of OBP48 homodimer located at the interface between the two OBP48 monomers.

A number of potential ligands were also identified by virtual screening based both on ligands whose binding specificities had been experimentally determined for all OBPs investigated by ENAROMaTIC and/or all the structures and homology models outlined above.

The virtual screening protocol involved large chemical databases, both commercial and public ones. As a result of this effort, specific databases listing potential ligands for all examined OBPs are currently available, which include ligand binding characteristics, such as estimated free energies of binding, Kd's and binding probabilities for each OBP that was included in the database as well as toxicity indices for the listed compounds.

Parental leads (compounds actually shown to represent bioactive ligands for selected OBPs), selected compounds identified by virtual screening and either purchased or synthesized in the context of the ENAROMaTIC project (see section 6 below) together with a small set of compounds, which were provided by one of ENAROMaTIC's industrial collaborators and confirmed theoretically for capacity to bind to specific OBPs, were evaluated experimentally for binding to the specific OBPs and *in vivo* functionality. As outlined earlier in this report (sections 2 and 4 above), the experimental results confirmed, to a large extent, the predictions made by the modelling studies.

These studies confirm in an unequivocal manner that the ligand- and structure-based approach could facilitate the discovery and design of novel repellents with enhanced binding selectivity (shape complementarily) and affinity. Repellents with enhanced affinity for OBPs can be used in lower concentrations and be detected over longer distances, therefore could be incorporated into time-release systems that deliver active ingredients into the air (spatial repellents). Hence, they will protect a living space in its entirety and the need for direct application of repellent to the body being avoided. This is important for those mosquitoes— *A. gambiae* in particular— that prefer indoor environments and human hosts.

6. *Synthesis and testing of ligand derivatives*

Ligand derivatives containing specific side groups relative to a "parental" ligand of known binding specificity for two specific OBPs and two ORs have been tested in order to assess the importance of the modifications to the fit of a ligand into the binding pocket of the specific OBPs and ORs. Thus, based on the observation that in *A. gambiae*, a human sweat component, *p*-cresol, mediates electrophysiological activity in OR1 and that *o*-cresol produces OR2-mediated activity, these two volatile compounds were chosen initially for study. Techniques for preparing radiolabelled versions were optimised and then radioactive *o*- and *p*-cresols were prepared by catalytic reduction of the modified (halogenated) cresols with tritium gas. The crude labelled products were purified and their radiological activities determined. The stability of the compounds was also confirmed.

Attempts were made to show that expression of OR1 and OR2 together with OR7 (Orco) would enable binding to the tritiated ligands *o*- and *p*-cresols and indole. However, no binding could be detected. Since it is known that these compounds should interact with the ORs it was concluded that the failure must be due to either the nature of the radiolabelled analogs, the assay conditions or the inability of the receptors to adapt a conformation that can bind a ligand. Attempts to show binding of the radiolabelled ligands to OBP1 and OBP3, which are known to bind the non-radioactive ligands, were also unsuccessful.

Given the progress of the crystallization and structure- and homology-based studies described under section 5 above, other non-radioactive derivatives of known ligands were synthesized. Specifically based on the deduced structural details of the OBP1–DEET and OBP1-indole complexes, a number of DEET and indole derivatives were prepared for structural and *in vivo* studies. The predicted binding affinities of the newly made compounds for two specific OBPs have been predicted to be significantly higher than those experimentally determined for DEET and indole. Binding experiments involving the selected OBPs and the first examined derivative compound, indeed revealed binding affinities much stronger than those for DEET. Interestingly, cell-based assays using insect cells expressing different ORs have also shown that the specific DEET analog acts as an OR antagonist suppressing OR activity to an extent considerably higher than that of DEET. Despite this, however, behavioral tests on live laboratory mosquitoes did not produce the anticipated enhanced repellence effects. This unexpected finding has been attributed to a reduced volatility of the derivative compound but this hypothesis requires experimental testing for

validation. While the analysis of the remaining compounds is still incomplete, the results obtained thus far indicate that addition of side chemical groups on ligands and their positions in the molecules may affect profoundly ligand binding and bioactivity properties in a positive or negative fashion.

Finally, the complementary approach of mutagenizing residues of the OBP's binding pocket and deducing the consequences on known and derivative ligand binding has also been carried out as described under section 5 above. Specifically, multiple mutated versions of two OBPs containing specific amino acid changes in their ligand binding pockets were generated and binding studies were undertaken using indole derivatives with an additional methyl group in a variety of positions as well as compounds that featured different side chains with varying numbers of carbon atoms present between the indole trunk and a terminal extension carboxylic acid as potential ligands. This work is still in progress.

7. *Evaluation of lead compounds for cytotoxic effects*

The aim of this objective was to undertake a safety evaluation of newly discovered olfactory disruptors through the use of neuronal network microelectrode array biosensors. Mammalian neuronal network microelectrode array (MEA) neurochips were used for this purpose to establish the lack of cytotoxicity of the selected ligands. Cultures of rodent cortex and midbrain cells cultured on the surface of MEA neurochips were set and analyses of neuronal network electrical activity patterns were determined upon acute application of two widely used repellents, DEET and EBAAP. These initial studies suggested that the two compounds could be used as reference compounds for newly discovered ligand leads as they cause no cytotoxic effects at the concentrations used for effecting their action. To expand the collection of standards, four pesticides, five compounds characterizing the acetylcholine receptor and four compounds characterizing the dopaminergic system, which are known to cause specific changes to neuronal cells were also analyzed and the electrical activity patterns of the responding cells established.

Subsequent testing of four most active of the newly discovered natural repellent compounds on the cortical networks was carried out using as standards the 15 compounds mentioned above. All four odorant compounds were found to cause cell activity loss at concentrations of 300 μM suggesting that none of the examined compounds are toxic unless ingested in considerable quantities.

The same repellent compounds have also been tested for acute cytotoxic effects on midbrain networks. Independently, the four compounds caused loss of activity of the cultures at concentrations lower than 200 μM. Mixtures of the same repellent compounds were also examined for acute cytotoxic effects on midbrain networks. The combination of repellent pairs, each at concentrations equal to those examined singly, induced no additional or synergistic effects compared to the single odorants. The combination of three odor compounds in a ratio of 1:1:1 shifted the EC50 produced enhanced acute effects relative to those of the single compounds.

Overall, the results of the acute experiments indicate that, if not applied orally, all new repellent compounds should be non-hazardous to mammals below concentrations of 100 μM. Caution is advised, however, because the data also suggests that these compounds could be toxic if taken orally at high concentrations. The toxicity of two of the new repellent compounds is comparable to those of DEET and EBAAP, while two other repellents appear to be safer to use than DEET and EBAAP.

The same repellent compounds were also examined for their chronic effects on midbrain and frontal cortex networks by repeated doses over 7 cellular divisions. Based on

comparisons that involved 60 different activity parameters, two of the tested repellents were shown to induce activity changes in the midbrain network similar to those of DEET and should therefore be considered as slightly neurotoxic requiring caution in use, while the other two caused no impairment of the midbrain network activity.

For the frontal cortex MEA culture system, electrophysiological experiments to assess chronic neurotoxicity have been performed only with DEET (at 300 μM) and EBAAP (at 200 μM). The results suggest that these compounds taken orally could be toxic at high concentrations.

In conclusion, while caution is certainly advised for the use of the newly discovered repellent compounds, these compounds do not seen to be more toxic that the two widely used commercially available repellents DEET and EBAAP.

8. ***Field testing of the most promising disruptors***

The disruption of host seeking behavior of female mosquitoes could provide new effective tools to reduce the incidence of human vector contact and disease transmission. The main objective of this work package is to evaluate the efficacy of new olfactory disruptor compounds on the behaviour of female *Anopheles gambiae* under (i) experimental hut and (ii) field conditions.

Accordingly, the first studies in a chosen site in Nigeria have been carried out that involved (i) the establishment of model huts to be used for the initial analyses of the effectiveness of the consortium's most promising lead compounds under semi-field conditions and (ii) an assessment of mosquito vector dynamics in the huts relative to the general geographic area where the huts are located.

Because normal village dwellings vary considerably in sizes, construction and amount of paraphernalia, which make mosquito collection difficult, experimental huts of standard shape and size are required for establishing initially mosquito behaviour under natural condition. For this reason, six experimental huts fitted with veranda and window exit-traps were built according to WHO guidelines and put into operation in 2010 in Nigeria where *A. gambiae* and malaria are endemic. Host seeking wild mosquitoes (anophelines and culicines) were able to enter and exit the hut via open eaves on three sides of each hut. The eave on the other side opened into a screened veranda that trapped the mosquitoes. Inside each hut a human volunteer slept overnight with the test odour disruptor repellent mounted in an odour-dispersing apparatus.

The first results established the identity and distribution of different *Anopheles* species and molecular form populations in the huts relative to the surrounding area. No significant variations in mosquito attraction to the position of different huts were found. Also, the experimental setup appeared to be ideal for studying mosquito behaviour and evaluating new vector control tools under natural conditions.

The first efficacy assessments for four of the most potent new natural repellent compounds under experimental hut conditions were carried out initially in the experimental hut environment. Inside each hut a human volunteer slept overnight with the test odour disruptor repellent mounted in an odour-dispersing medium. For each hut, the number of mosquito entries, numbers trapped in the veranda and window exit traps and numbers resting inside each hut were recorded over a period of two months. The efficacy of the four odour disruptor compounds that proved effective under laboratory conditions and safe for humans was assessed in relation to DEET (a standard repellent). One compound showed repellency equivalent to the best reference compound (DEET) used as comparison standard, while binary and ternary mixtures of candidate compounds produced significantly enhanced repellency

effects. Specifically, the results from the experimental hut revealed that only one out the four odour disruptors has efficacy equivalent to DEET. However, the binary mixture of the two compounds that ranked #1 and #2 in terms of repellence potency produced an enhanced repellent effect on both *Anopheles* and *Culex* mosquitoes compared to DEET.

Once this proof of principle was established, real field (village) applications of the specific "olfactory disruption model" were evaluated over a period of 22 nights in a selected Nigerian village by placing odour disruptor compounds inside 10 randomly selected houses. The study area had the two most widely distributed Afrotropical malaria vectors (*Anopheles gambiae s.s*: 60% and *An. arabiensis*: 40%) and an ideal set up for evaluating potential malaria vector control tools.

The village study showed that the number of mosquitoes of any species entering individual rooms with odour disruptors was not statistically different indicating that for all repellents examined there was a limitation in terms of the distance over which they could be perceived by the mosquitoes. However, once the mosquitoes had entered a room, significantly more *Anopheles* were repelled by the odour disruptors than *Culex*. The most active natural repellent repelled 2.0-2.4 times more *Anopheles* and 1.7-2.3 times more *Culex* mosquitoes compared to the other three compounds and was shown to be the only repellent with activity similar to DEET. As with the case of the experimental hut testing, the study of the binary and ternary mixtures of the natural repellents revealed enhanced repellence effects relative to the single compounds and DEET.

It is not known what magnitude of difference such a degree of repellence could translate into in terms of detectable levels of vector control or reduction in disease transmission. However, the experimental hut and village platform with free flying mosquitoes enabled observations of more natural interactions between female mosquitoes and odour disruptor compounds and have provided a better indication of the efficacy of odour disruptors in real life situations.

Ethical Issues

All compounds employed in field tests were thoroughly examined for their safety on humans and other mammalian species in accordance with section B4.1 of Annex I. As has been elaborated under section #6 above, these compounds have been evaluated for mammalian neurotoxicity using a highly sensitive neuronal network microelectrode array system for rodents and found to lack mammalian neurotoxicity.

As was also scheduled, none of the compounds tested for field efficacies as potential mosquito repellents came into contact with humans or nearby animals. Their effectiveness has been evaluated through the use of mosquito traps, which are normally used in disease endemic countries for the purpose of determining local mosquito population densities through counts of the numbers of trapped mosquitoes.

All volunteers participating in the tests in experimental huts were provided with malaria prophylaxis prior to the start of the work and were also provided with mosquito nets in the experimental huts. Furthermore, ethical clearance for the specific field studies has been obtained from the Ethical Review committee of the Nigerian Institute of Medical Research before starting work on the relevant work package.

Description of potential impact including the socio-economic impact and wider societal implication of the project so far; main dissemination activities; and exploitation of results

Introduction

With almost 400 million clinical cases and approximately 1 million deaths annually (the majority of the victims being children under the age of five), malaria, which is transmitted to humans by the African malaria mosquito vector *Anopheles gambiae*, represents one of the most serious infectious diseases of the world, particularly for under-developed and developing countries. Additionally, other mosquito-born infectious diseases that had been eradicated in the industrialized world are now making a come back due to climatic changes, increased frequency of air travel and high influxes of immigrants from third world countries. During the last two years, for example, repeated incidents of *Plasmodium vivax*-caused malaria transmitted by local Anopheline mosquitoes have been reported in Greece. In addition, West Nile virus encephalitis transmitted by local strains of *Culex pipiens* mosquitoes and, in 2012, Dengue virus haemorrhagic fever transmitted by local *Aedes* mosquitoes have also been reported.

Over the years, various European countries and the EU, as a political entity, have contributed significant aid in support of national (in malaria-endemic countries) and international efforts aimed at alleviating the impact of the disease on its victims and controlling its spread through improvement in local environmental and living conditions. This has included measures aimed at the control of the mosquito populations that vector the disease. However, despite the fact that huge efforts have been devoted to date in order to provide means that might stem the spread of the disease, the continuing lack of effective drugs and vaccines and the rise of resistance even to the newest generations of insecticides in the mosquito vector populations have represented important adverse factors prohibiting effective control of the disease. At a European level, coordinated efforts aimed at the development of multidisciplinary and integrated approaches for vector control or interference with vector capacity to transmit the disease to human hosts only recently started to materialize with attention being primarily focused on the need to overcome the emergence of insecticide resistance.

An additional and largely unexplored area of scientific investigation that promised to provide powerful new solutions to the sought after goal of malaria control, has been mosquito olfaction and the possibility of discovery and development of effective and environmentally safe, tools to prevent female mosquitoes from locating human hosts and transmitting disease agents like malaria parasites during blood feeding. Repellents can act as a first line of personal defence against mosquito bites and thus keep female mosquitoes bearing infectious agents away from humans and prevent an infected human from spreading parasites to uninfected mosquitoes. However, the prohibitive cost of conventional repellent discovery by industry - it takes about 10 years and $30 million to develop a new repellent with conventional methods used by industry - has prevented the development of new repellents over the years. As a result, the current number of repellents that protect effectively against mosquito bites is astoundingly small with N,N-diethyl-m-toluamide (DEET), a chemical discovered in 1946, being still the most widely used repellent despite its disadvantages due to the general lack of effective alternatives.

ENAROMaTIC has been the first consortium to materialize in Europe with a mandate to coalesce into a multidisciplinary scientific consortium many European groups involved in the study of insect (including mosquito) olfaction, which until then had been working at a basic

level without necessarily being particularly focused on the achievement of practical measures for the prevention of transmission of malaria. This multidisciplinary consortium, which commanded important methodological expertise from diverse scientific fields, also contributed all required infrastructure and focused primarily on issues related to olfaction-induced behaviors of mosquitoes and malaria transmission.

The scientific impact: a new paradigm for prevention of mosquito-born infectious diseases established by ENAROMaTIC

As detailed below, the strong collaborative interactions forged amongst the ENAROMaTIC project participants, have produced results that demonstrated the feasibility of increasing the efficiency and impact of European research on mosquito olfaction both at the level of basic research and that of technology transfer. In turn, this outcome allowed ENAROMaTIC to reach the goal of discovering and using specific volatiles as effective mosquito olfactory disruptors for control of transmission of mosquito-borne infectious diseases.

In the four years of its life, the ENAROMaTIC project, which targeted the mosquito vector *A. gambiae*, achieved the development and deployment of rational, target-based bioassays that permitted the identification of powerful natural repellent compounds, which, when placed at a distance from human subjects, induced mosquitoes to exit rooms they had previously entered. More specifically, ENAROMaTIC used subsets of *A. gambiae* odorant binding proteins (OBPs) and odorant receptors (ORs) expressed predominantly in female antennae as specific targets for identification of compounds of synthetic or natural origin capable of effecting changes in the normal responses of mosquitoes to odor plumes of human origin. For the OBPs, the screening assays allowed the identification of both synthetic chemicals and constituents of essential oils (EOs) capable of binding to them *in vitro*. With subsequent analyses of the OBP binding oils using specialized behavioural and electrophysiological methods in conjunction with techniques for chemical compound identification, several EOs and individual constituents displaying strong repellent activities were identified. Thus, by using such rational approaches, which included parallel analyses for modulation of mosquito OR function, the rate of discovery of compounds with repellent activity was accelerated substantially relative to the random approaches employed by industry.

As indicated above, the approaches implemented by ENAROMaTIC resulted in the identification of several essential oils and constituent compounds displaying strong repellent activities for *A. gambiae* mosquitoes. Four of the strongest natural repellents were tested for safety and found to be lack neurotoxic and cytotoxic activities; and field-testing in Africa showed two of them and blends thereof to be effective spatial repellents that matched and, in some cases, outperformed DEET, the most widely used olfaction standard, in effectiveness. Additionally, more than 150 EOs obtained from plant families different than those of the initial group, were examined and several were found to contain OBP binding and repellent activities of as yet unknown but easily identifiable constituents. Most importantly, the work of ENAROMaTIC has shown that natural repellents developed against Anopleline mosquitoes were also very active against *Culex* mosquitoes that vector the West Nile, Japanese and St Louis encephalitis viruses, and even, but to a lesser extent, against sand flies, vectors of *Leishmania* parasites.

Last but certainly not least, the consortium's studies on OBP structure determination have confirmed that ligand- and structure-based modeling approaches could facilitate the design of novel repellents with enhanced affinity for OBPs that may be used in lower concentrations

and be detected over longer distances and, therefore, be incorporated into release systems that deliver active ingredients into the air (spatial repellents) over significant periods of time. This is particularly important for endophilic mosquitoes like *A. gambiae,* which prefer indoor environments.

These achievements represent major advances for the field and constitute a new paradigm for mosquito and infectious disease transmission control. As such, the achievements have expanded the arsenal of available means for vector control and contributed toward a transition to a more comprehensive integrated approach to pest management and infectious disease prevention control. Other than establishing an overall proof of principle in its approach, the ENAROMaTIC consortium, has clearly contributed to the "European Research Area" in a new and highly relevant field of research and development. The consortium has forged strong interactions that will likely last long beyond the lifetime of this specific initiative and ensure that Europe continues to hold its place on the world stage of insect olfactory research.

The socio-economic impact and wider societal implications of the project

For the mosquito malaria vector, the unique approach pursued by ENAROMaTIC has resulted in the identification of disruptors of the host seeking behavior of female mosquitoes from natural sources and, thus, provided new tools to be employed in the effort to reduce the incidence of contact between the human host and the insect disease vector and a reduction in the spread of malaria transmission. Because malaria is such a pervasive plague in Africa, Central and South America and South East Asia and the control of the vector population such a formidable task, it was clear at the outset that the initial impact of the research would occur mainly in countries where malaria is currently endemic. This was also the reason why the field tests that assessed the effectiveness of the newly discovered natural repellent compounds were undertaken in an African country where the malaria mosquito vector and the disease are endemic. However, given the global warming caused by the climatic change and the consequent spread of malaria mosquito vectors in what used to be more temperate climatic zones, the need for malaria transmission control may in the foreseeable future become relevant to Europe and other countries where malaria had been previously eradicated. Moreover, the ENAROMaTIC finding that at least some of the newly-discovered compounds of plant origin act as powerful repellents not only for *A. gambiae* but also for other hematophagous insect disease vectors such as female *Culex* mosquitoes (vectors of other infectious diseases including West Nile virus, filariasis, Japanese encephalitis and avian malaria) and female sandflies (responsible for the transmission of *Leishmania infantum chagasi* parasites) increases further the potential impact for the consortium's discoveries.

As already indicated, the isolation of bioactive compounds capable of acting as insect pest control agents from natural sources, particularly essential oils, is receiving increased attention at present due to (i) the enormous diversity of compounds that such renewable natural resources contain, which can now be examined for desired bioactivities using novel biotechnological approaches such as those employed in the proposed work; and (ii) the increased probability that many natural compounds with desired bioactivities will act not only effectively but also in a safe fashion that will not burden human health and the environment with unwanted pollution and toxic side-effects, thus satisfying the public's requirements for the development of products of natural origin derived from renewable natural resources. Therefore, the anticipated discovery of new substances of botanical origin having mosquito repellent or attractant properties is crucial, because their industrialization for production of new biocide products, which will be devoid of non-biodegradable chemicals or

compounds dangerous for the Public Health, is expected to bring significant benefits to society and the environment. It is for such reasons that the ENAROMaTIC project may not only produce specific value-added applications that will be of great benefit to the countries afflicted by malaria but also provide significant economic benefits to the European economy. Specifically, the results achieved by ENAROMaTIC should foster the competitiveness of Europe's biotechnology industry by encouraging industrial partners to adopt technologies optimized or developed by the consortium in a collaborative and integrated fashion with the relevant academic centres. The interest displayed by our industrial collaborators and other industrial entities primarily in Europe and the USA in response to Press Releases announcing the availability of the new natural repellents for licensing attest to this assessment.

Main dissemination activities

For the scientific community outside the consortium boundaries and the public at large, caution was exercised to disseminate results with commercial potential after protection of patentable intellectual property had been ensured. For scientific results, the dissemination effort included (i) publication as normal scholarly literature in high impact scientific journals of general interest as well as insect-specific and applied science journals; (ii) presentations at national and international conferences. Importantly, ENAROMaTIC, in collaboration with the European Commission and consultation with other EU-funded projects on malaria mosquito vector and transmission control, undertook the organization of the second conference on "Malaria vector biology and transmission control", which took place in Crete, Greece in the summer of 2011 and became also the venue for the assessment of ENAROMaTIC's activities by an international panel of experts that formed the advisory and evaluation board of the consortium and also contributed to the strategic orientation of future EU-funded vector and transmission research.

For Industry and other interested parties related to exploitation, dissemination took the form of Press Releases that were followed by non-confidential discussions in anticipation of completion of the process of protection of patentable intellectual property.

Finally, general information about the project and its aims and achievements was also presented to the public at large in the public access part of the ENAROMaTIC website and also by giving interviews in mass communications media (radio, television, newspapers and magazines of general interest) and issuing relevant press releases for breakthroughs deemed to deserve wider publicity.

Intellectual property protection and exploitation

ENAROMaTIC had developed an Intellectual Property (IP) Agreement, which contained all IP-related issues and general principles on approaches to be taken for matters relevant to this domain. This Agreement, which was signed by all partners and became part of the Consortium Agreement, anticipated the following:

- Identification of patentable innovations

Partners were to communicate on a confidential basis in order to evaluate the potential of new products or technologies for possible patent filing or other means of protecting intellectual property. The project policy was that patenting of results to be exploited should occur without undue delays that could prevent rapid publication of results and circulation of information among partners.

- Ownership of knowledge

As detailed in the model contract for FP6, intellectual property belongs to the partner developing the concept and making the discovery. Protocols for joint ownership were also identified in the consortium agreement. Each partner identified and defined pre-existing intellectual property rights (IPR), which were available for the project and those that could not be made available on a royalty-free basis or had other specific restrictions for use by the consortium members. All partners also declared pre-existing know-how to be contributed to or introduced into the project.

- Protection of knowledge, access rights for further research and licensing options

The consortium Agreement anticipated access rights for use of the intellectual property for research activities on a royalty-free basis. Irrespective of ownership, there was an obvious imperative that all consortium partners who needed access to the developed technology should receive the necessary licences for research use from the owners. The IPR agreement provided for this, but did not include a blanket cross-license to all parties. In general, findings generated by one participant or one working group within the consortium that were required by another participant for conduct of their work under the project were made available on a royalty-free basis for intellectual property generated during the course of the contract, and, unless otherwise agreed before signing the contract, pre-existing know-how as well. Findings generated by other participants that were needed by a given participant in order to exploit knowledge, which he/she generated within the course of the contract, were royalty-free for intellectual property generated during the contract and non-discriminative conditions applied for intellectual property created outside the project during its duration.

The strategy for IP protection anticipated application filing to a national or the European Patent Office for the purpose of acquiring priority date and subsequent PCT filing for international patent protection, as judged appropriate within a year from the initial filing. The strategy also anticipated that, should this be deemed necessary, the USA partner to also contribute to the protection process and subsequent exploitation effort.

- Use and dissemination of knowledge and exploitation of intellectual property

All partners who have contributed to the creation of intellectual property should share in fair and reasonable commercial returns should the intellectual property be exploited. Universities within the consortium might wish to see industrial collaborators of the consortium as the preferred route to commercialise, provided these collaborators have the interest, resources and base to undertake the commercialisation.

For the cases that the IP owner(s) sought to commercialise, the financial reward would follow inventorship (and hence ownership) with specific revenue sharing agreements made between the owners. The consortium did not advocate a wider revenue sharing obligation, as this has been judged to represent a major disincentive for commercial exploitation by the private sector, particularly SMEs. Other key points were that all pre-existing intellectual property at the start of the consortium remained in the ownership of the party that introduced it. There would be no obligation to license such existing intellectual property to any Consortium member, unless a clear need existed for this to happen in order for project objectives to be met. Similarly, the IPR agreement acknowledged that partners may be working in the same area outside of the Project and that independently generated intellectual property would not be part of the project intellectual property, though individual Consortium members might introduce such intellectual property at any time as they saw fit.

Decisions of whether and how to proceed for IP protection were to be made collectively by the inventors. For example, if newly identified compounds were already protected by a patent

for a different or similar types of action, the inventors' claims would be based on the action of specific mixtures (blends with other natural compounds) and formulations containing the active compounds.

Given the effectiveness and safety properties of the specific natural repellents developed by ENAROMaTIC partners and the anticipation that such repellents would find a strong competitive niche in the market and thus be attractive to commercial interests, the effort for the initial protection and commercialization of the relevant IP in Europe and the USA has been initiated by the Technology Transfer Office of one of the European partner institutions of ENAROMaTIC in accordance to the principles outlined above. The commercialization effort targets two distinct but interrelated directions, (i) licenses for manufacturing and marketing mosquito repellent formulations containing single repellent compounds or compound mixtures known to lack neurotoxicity and general cytotoxicity activities; and (ii) for other essential oils and fractions thereof shown to contain strong repellent activities of an as yet unknown identity, and the signing of agreements for provision of information to interested commercial entities, which would explore further such repellent oils for the purpose of identifying constituents displaying the repellent properties and developing additional products for the market.

Dissemination of information by the exploitation agency regarding the qualities and advantages of the newly-developed products to appropriate target groups including end users and Industry in industrialized countries, policy-makers and specific interest groups in third world countries, the media and the public at large worldwide would be crucial success factors. It should be also noted that a special effort will be made to find commercial entities that have the required know-how and be willing to exploit the development of such repellent products with special marketing and pricing considerations that would also promote sales in third world countries afflicted by malaria and not only in the industrialized world. The requirements for such product development will include formulations that would give desirable qualities to the marketed products, such as enhanced stability in adverse climates, controlled release rates for prolonged action and means of dispensing appropriate for different parts of the world, even in locations where the use of electricity is precluded. Such considerations should be coupled to appropriate marketing policies that would make pricing of products affordable for countries of the third world.