# Genome-wide analysis of transcriptomic divergence between laboratory colony and field *Anopheles gambiae* mosquitoes of the M and S molecular forms

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## Abstract

**Our knowledge of *Anopheles gambiae* molecular biology has mainly been based on studies using inbred laboratory strains. Differences in the environmental exposure of these and natural field mosquitoes have inevitably led to physiological divergences. We have used global transcript abundance analyses to probe into this divergence, and identified transcript abundance patterns of genes that provide insight on specific adaptations of caged and field mosquitoes. We also compared the gene transcript abundance profiles of field mosquitoes belonging to the two morphologically indistinguishable but reproductively isolated sympatric molecular forms, M and S, from two different locations in the Yaoundé area of Cameroon. This analysis suggested that environmental exposure has a greater influence on the transcriptome than does the mosquito's molecular form-specific genetic background.**

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## Introduction

The mosquito *Anopheles gambiae* is the primary vector of *Plasmodium falciparum*, the causative agent of human malaria in sub-Saharan Africa. This mosquito species transmits malaria between humans across a wide variety of ecological settings throughout Africa (Gillies & de Meillon, 1968; Coetzee *et al.*, 2000). This ecological plasticity is reflected, at the genetic level, by large numbers of molecular and chromosomal polymorphisms that provide great evolutionary potential as a reservoir of genetic variability (Coluzzi *et al.*, 2002; Pombi *et al.*, 2008; Costantini *et al.*, 2009). The recent development of genomic and functional genomic approaches in malaria vector research, together with the feasibility of rearing this mosquito species in the laboratory, have fostered the use of this model system to explore the physiology, genetics and evolution of anopheline vectors of malaria. Several major breakthroughs in our understanding of various facets of mosquito biology such as insecticide resistance, immunity and vector-parasite interactions have resulted from these studies (reviewed in Barillas-Mury & Kumar, 2005; Enayati *et al.*, 2005; Michel & Kafatos, 2005; Chen *et al.*, 2008; Yassine & Osta, 2010). The mosquito immune system plays a key role in its interaction with the malaria parasite *Plasmodium*, and a variety of mosquito effector molecules have been shown to either facilitate or prevent infection in the mosquito (Dong *et al.*, 2006, 2009; Mendes *et al.*, 2008; Dong & Dimopoulos, 2009; Garver *et al.*, 2009; Jaramillo-Gutierrez *et al.*, 2009; Mitri *et al.*, 2009; Volohonsky *et al.*, 2010). However, our current knowledge of the general molecular physiology and immune system of *A. gambiae* is almost exclusively based on studies performed using laboratory strains that have been selected and maintained under laboratory conditions for the past 15–30 years (Cohuet *et al.* 2006; Aguilar *et al.*, 2005;

Tripet, 2009). These laboratory colonies are likely to have diverged significantly from the field populations from which they originated, as a result of strong founder effect at the onset of the colony, enhanced genetic drift and inbreeding in small caged populations and/or selection for some specific trait such as the ability to feed on artificial blood sources (Tchuinkam *et al.*, 1993), susceptibility/ refractoriness to parasite infection (Collins *et al.*, 1986; Vernick *et al.*, 1995) and/or susceptibility/resistance to insecticides (Müller *et al.*, 2007). The colonization process together with limited environmental variance under laboratory conditions and standardized rearing methods have led to limited genetic variation, which in turn results in a dramatically reduced phenotypic variance in lab-reared colonies (Tripet *et al.*, 2008; Tripet, 2009). For example, studies in *Drosophila* have indicated that inbred populations have lower fitness (or stress resistance) and are less adaptable than are the outbred populations in the field from which they were derived (Frankham *et al.*, 2000; Hoffmann *et al.*, 2001; Woodworth *et al.*, 2002; Reed & Frankham 2003; Reed *et al.* 2003). It has also been shown that microsatellite DNA polymorphisms in *A. gambiae* are dramatically reduced in laboratory colonies when compared to field populations (Norris *et al.*, 2001). Hence, inferences generated from studies using non-natural model systems and/or inbred colonies of vectors (and parasites) may be limited and only reflect some basic characteristics of the natural systems, prompting for subsequent validation in real-life situations. Here, we compared gene transcript abundance profiles between one laboratory colony of *A. gambiae* and two field populations from the vicinity of Yaoundé, Cameroon, to explore the divergence of their global transcriptomes.

*Anopheles gambiae* is the nominal member of a species complex that groups together seven named species of relatively recent and rapid origin, all of which are closely related, sharing considerable genetic variation and being morphologically indistinguishable from one another (Powell *et al.*, 1999; Coluzzi *et al.*, 2002; Besansky *et al.*, 2003). Diversification and further radiation of most species within the *A. gambiae* complex have been suggested to be driven by divergent ecological selection, allowing colonization of specific larval development sites, thus fostering assortative mating and speciation (Coluzzi, 1982; Ayala & Coluzzi, 2005). Within *A. gambiae*, the process of ecological divergence and lineage splitting is ongoing (Lehmann & Diabaté, 2008; Manoukis *et al.*, 2008; Costantini *et al.*, 2009; Simard *et al.*, 2009) and recent studies have subdivided natural populations of *A. gambiae* into two morphologically identical and broadly sympatric molecular forms, called the M form and the S form, which are reproductively isolated and genetically different at several DNA loci, reflecting barriers to gene flow (della Torre *et al.*, 2001, 2002, 2005; Turner *et al.*, 2005; Lehmann &

Diabate, 2008; White *et al.*, 2010). Although interbreeding between the M and S forms yields fertile progeny (Diabaté *et al.*, 2008), MS hybrids are rarely observed in nature; when these forms overlap in time and space, the rate of heterogamous insemination is only ~1% (Tripet *et al.*, 2001), clearly demonstrating the existence of a pre-mating barrier. Thus, both indirect and direct genetic evidence indicate incomplete but substantial barriers to gene flow between the two molecular forms of *A. gambiae s.s.*, pointing to the very earliest stages of speciation (della Torre *et al.*, 2002; Costantini *et al.*, 2009). A significant body of knowledge on the genetic differences and geographical distribution of the M and S forms has been established (della Torre *et al.*, 2005; Lehmann & Diabate, 2008; Costantini *et al.*, 2009; Simard *et al.*, 2009; White *et al.*, 2010), but the variations and differences in their physiology and the genes and pathways involved in local adaptation and speciation are still largely unidentified. In Central Africa, both molecular forms are sympatric and can be found together in the same larval development sites. However, the M molecular form is more prevalent in urbanized/polluted areas, habitats of marginal quality, whereas the S form seems to predominate in more rural settings (Kristan *et al.*, 2003; Carrara *et al.*, 2004; della Torre *et al.*, 2005; Wondji *et al.*, 2005; Simard *et al.*, 2009). Here, both molecular forms are chromosomally homosequential at the cytogenetic level, showing mainly standard chromosomes without inversions (Pombi *et al.*, 2008; Simard *et al.*, 2009), offering opportunities to explore genetic and physiological differences between molecular forms without the confounding effect of chromosomal inversions.

Regulation of transcript abundance plays a key role in determining the fitness of a genome, which is critical for development and adaptation. The ability of a population to adapt to the environment is dependent on its phenotypic diversity, which in turn is a consequence of genetic diversity. Polymorphism in the pattern of gene transcript abundance is a widespread phenomenon and has been observed in bacteria (Cooper *et al.*, 2003; Le Gall *et al.*, 2005), yeast (Townsend *et al.*, 2003), mice (Schadt *et al.*, 2003) and humans (Yan *et al.*, 2002). Although most of this variation may be neutral and reflect genetic distance amongst taxa/populations, several recent studies have demonstrated that natural selection through environmental pressure is a determinant of at least part of this polymorphism in gene transcript abundance (Enard *et al.*, 2002; Oleksiak *et al.*, 2002; Cooper *et al.*, 2003; Ranz *et al.*, 2003; Townsend *et al.*, 2003; Ogura *et al.*, 2004; Le Gall *et al.*, 2005; Giger *et al.*, 2006; Whitehead & Crawford, 2006). These particular studies suggest that long-term behavioural differences are a result of substantial physiological remodelling which, in turn, is guided by the transcriptome. Natural selection can lead to mutations in

*cis-* and *trans*-acting transcriptional control elements that promote divergence in transcript abundance (Denver *et al.*, 2005; Rifkin *et al.*, 2005). Consistent with these predictions, at the early stages of speciation, as in the case of the M and S molecular forms of *A. gambiae*, divergence between incipient species might be more easily detectable at the transcriptome level, before reciprocal monophyly is established at the molecular level.

Despite the profound influence of the transcriptome on the divergence that occurs between individuals and populations exposed to different environmental conditions, most studies have addressed divergence only at the genome sequence level. To date, a single study by Cassone *et al.* (2008), employing the Affymetrix Gene-Chip platform, has investigated differences in gene transcript abundance between laboratory colonies of the M and S molecular forms and showed ~1–2% of their genes as differently expressed, with a strong bias toward transcription- and sensory process-related functions. In the present work, we compared the transcriptome within and between the two molecular forms of *A. gambiae* collected from the same larval development sites in two distinct ecological settings around Yaoundé, in order to assess the degree of variation that can be attributed to the genetic background of molecular forms and to environmental conditions, respectively.

## Results and discussion

### Transcriptomic divergence between laboratory colony and field mosquitoes

To identify consistent transcriptomic differences between laboratory colony and field mosquitoes, we compared the global transcriptomic profiles of 4-day-old females of the *A. gambiae* Keele laboratory colony strain to those of the field S and M molecular form mosquitoes originating from two different locations in the Yaoundé area [the S molecular form collected at Nkolondom (A in Fig. 1) and the M molecular form collected at Nkolbisson (B in Fig. 1)]. The study was designed to identify transcriptomic differences that had resulted from continuous exposure over many generations (through natural selection) of these mosquito groups to different environmental conditions (laboratory vs. field), rather than differences that related to the immediate environmental exposure or life history of the individuals that were used for the assays. For this reason, we reared the field mosquitoes from the larval to the adult stage under insectary conditions identical to those of the laboratory colony mosquitoes. The observed differences in gene transcript abundance are thought to be a result of an accumulation of mutations that have influenced gene transcript abundance over many generations in mosquitoes that have adapted to either the field or the laboratory environment.
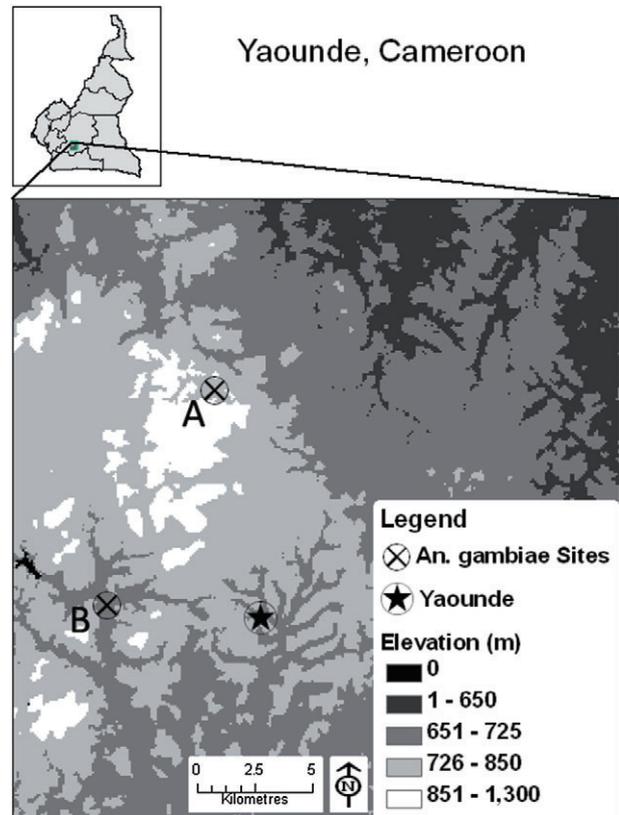


**Figure 1.** Geographical locations of the two sites from which field *Anopheles gambiae* were collected. A, Nkolondom; B, Nkolbisson.

A comparison between the transcriptomes of these laboratory colony and field mosquitoes showed that 518 genes displayed differential transcript abundance: 254 genes showed a higher transcript abundance in the field mosquitoes, and 263 genes showed a higher transcript abundance in the laboratory colony mosquitoes. Microarray-assayed gene transcript abundance was validated with real-time quantitative reverse transcription PCR (RT-PCR) for eight genes in the two sets of laboratory vs. field mosquito comparisons (Fig. 2). A tyrosine protein kinase (AGAP005763-RA) was the only gene displaying opposite transcript abundance patterns between the Nkolondom and Nkolbisson mosquitoes when compared to the laboratory mosquitoes (Fig. 3a′; Table S1). The transcripts showing higher transcript abundance in the field mosquitoes corresponded mainly to genes in the immunity, transport and cytoskeleton functional classes, with a large proportion belonging to the 'diverse' and 'unknown' groups (Fig. 3B). The fact that a larger number of immune gene transcripts were more abundant in the field mosquitoes is likely to reflect an adaptation of laboratory mosquitoes to a lower amount and diversity of microbes when compared to the microbial exposure of field mosquitoes. Amongst the transport genes that displayed a higher transcript abundance in the field mosquitoes, five have been putatively
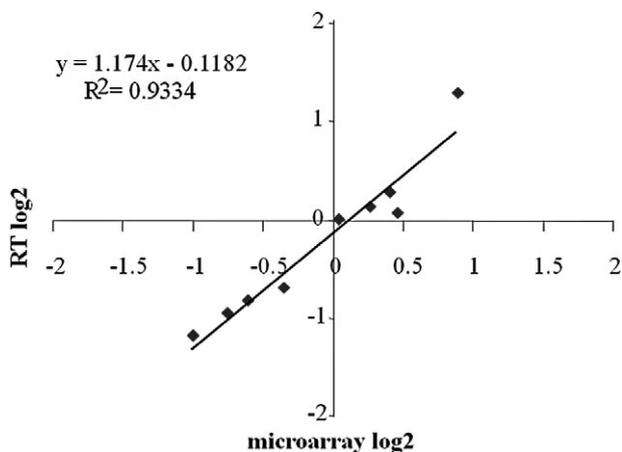
$$y = 1.174x - 0.1182$$
$$R^2 = 0.9334$$

**Figure 2.** Validation of microarray-assayed gene transcript abundance by real-time quantitative RT-PCR. Log$_2$-transformed microarray transcript abundance data (Nkolondom-S vs. Keele and Nkolbisson-M vs. Keele ratio) for eight genes were plotted against the log$_2$-transformed transcript abundance data obtained by real-time quantitative RT-PCR. The Pearson correlation coefficient ($r = 0.96$), best-fit linear-regression analysis ($R^2 = 0.93$) and slope of the regression line ($m = 1.174$) showed a high degree of correlation between the two assays in terms of the magnitude of the regulation. The individual values for all these genes are presented in Table S7.

linked to insecticide resistance (Roth *et al.*, 2003; Hemingway *et al.*, 2004): a multidrug-resistance P glycoprotein (ENSANGT00000028639), an ATP-binding cassette sub-family A member (ENSANGT00000025474), the secretory protein HlyD (ENSANGT00000000827), a proton-associated sugar transporter A deleted in neuroblastoma 5 (AGAP010856-RA), and a multidrug resistance-associated protein (ENSANGT00000028591; Table S1).

The field mosquitoes also displayed elevated transcript abundance of a larger number of structural and cytoskeletal components (Fig. 3B; Table S1). Eight sensory-related genes displayed differential transcript abundance between field and laboratory colony mosquitoes, presumably reflecting a certain degree of olfactory adaptation: Laboratory *A. gambiae* strains are routinely maintained on either mice or artificial membrane feeding systems, whereas wild *A. gambiae* are adapted to feed primarily on humans.

The laboratory colony mosquitoes showed elevated transcript abundance of several genes that are involved in oxidoreductive stress-related, mitochondrial, metabolic and replication-transcription-translation processes (Fig. 3B; Table S1). This increased transcript abundance may be linked to a generally elevated metabolic activity. Higher rates of protein synthesis require higher rates of anabolism and catabolism, which in turn require an elevated mitochondrial activity (Hochachka *et al.*, 1996). This higher translational activity could also explain the generally lower amount of other transcripts or could be a

consequence of a shift in resource allocation (Fig. 3B). An organism's protein pool is in a continual state of flux, with new proteins entering the pool as a result of protein synthesis and new proteins being removed as a result of protein degradation. This process is energetically expensive, but laboratory colony mosquitoes have adapted to live under optimal conditions without limiting factors (they are provided with food and mates *ad libitum,* and are largely free of pathogens), and it is possible that these optimal conditions have produced mosquitoes with higher metabolic rates and a stronger biosynthetic machinery.

### Transcriptomic comparison between the *A. gambiae* M and S molecular forms

The global transcriptomic profiles of 4-day-old females of the predominant molecular form from each location (M form in Nkolbisson, S form in Nkolondom) were compared to those of the other molecular form from the same location (Fig. 3C–E; Tables S2–S5). The similarity of gene transcript abundance was 10 times higher between the predominant forms from each location (61 transcripts in common between the M form from Nkolbisson and the S form from Nkolondom; Fig. 3C; Table S2) than within each molecular form (six transcripts in common between the M form from Nkolbisson and the M form from Nkolondom; Fig. 3D; Tables 1, S5), suggesting that adaptation leading to a dominant form under differing environmental conditions has a larger impact on the mosquito's physiology than does the molecular form itself. A similar pattern has been observed in two other studies: one on transcriptomic differentiation between sedentary and migratory trout (Giger *et al.*, 2006), and another on transcriptome variation affected by natural selection in the fish *Fundulus heteroclitus* (Whitehead & Crawford, 2006). The results of these studies suggested that the interaction of the genotype with the environment accounts for more transcriptional variation than does genetic ancestry.

The number of genes that was found to present different transcript abundance between the M and S molecular forms was 1.4 times higher in Nkolondom than in Nkolbisson (Fig. 3E; Tables S3, S4), suggesting that the environmental conditions in Nkolondom have a stronger differential effect on the physiology of each molecular form. A similar number of genes showed a higher level of transcript abundance in the two molecular forms from the Nkolondom location, whereas 63% of the differentially expressed genes showed a higher transcript abundance in the M molecular form than in the S form in the Nkolbisson area. In both locations, mosquitoes of the predominant molecular form displayed an elevated transcript abundance of genes that generally belonged to the immunity and chemosensory functional groups, whereas the less abundant molecular form displayed an elevated
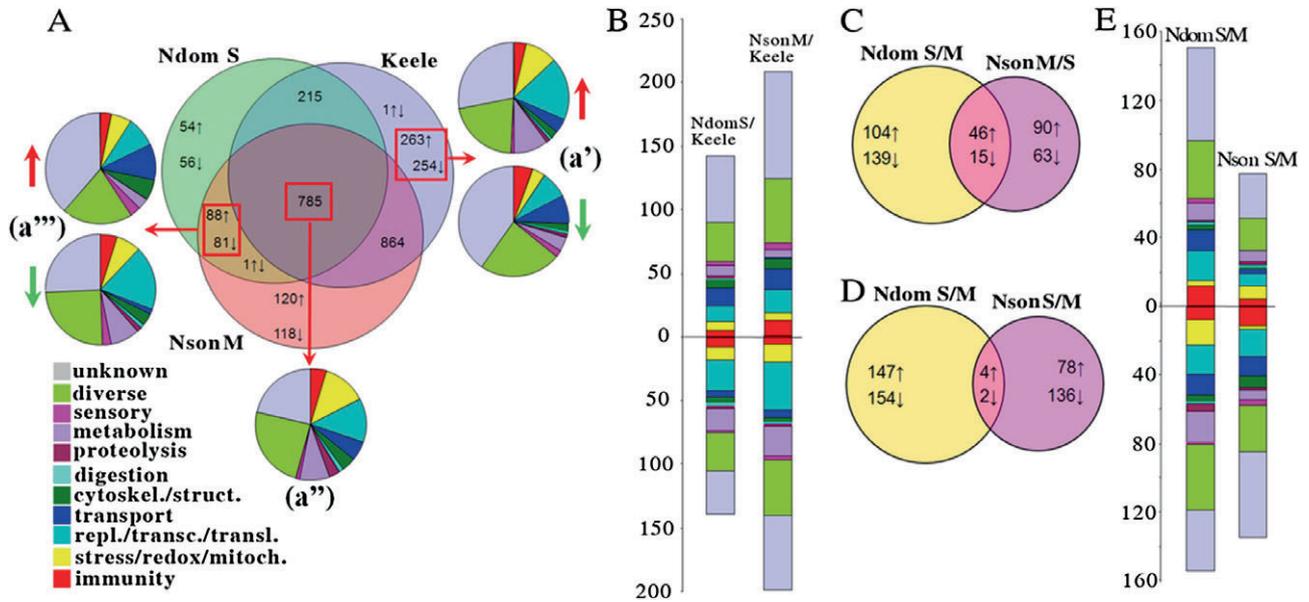
**Figure 3.** (A) Venn diagrams showing co-regulation and differential transcript abundance patterns for field and laboratory colony mosquitoes. (a′) Five-hundred and eighteen genes showed differential transcript abundance between laboratory mosquitoes and at least one of the two field strains (Table S1). Pie charts indicate relative proportions of functional gene groups that were represented by transcripts at higher and lower levels in laboratory colony mosquitoes than in field mosquitoes. The gene with arrows in both directions is a tyrosine protein kinase that showed higher transcript abundance in the Nson M strain and lower transcript abundance in the Ndom S strain when compared to the Keele strain. (a″) Seven-hundred and eighty-five genes showed similar transcript levels in field mosquitoes and laboratory colony mosquitoes (Table S8). The pie chart represents the proportions of the functional gene groups. (a‴) Those genes from section (a′) showing similar transcript abundance profiles in both field strains and differential abundance in laboratory mosquitoes are shown in the overlapping section: 88 genes showed higher levels of transcripts in the field mosquitoes, 81 had higher transcript levels in the laboratory colony mosquitoes, and one gene had a higher transcript level in the Nson M strain and lower level in the Ndom S strain when compared to laboratory colony mosquitoes (Table S9). The pie charts indicate the relative proportions of functional gene groups that showed transcript abundance at higher and lower levels in field mosquitoes than in laboratory colony mosquitoes. (B) Proportions of functional gene groups that displayed higher (above the horizontal line) or lower (below the horizontal line) transcript levels in field mosquitoes when compared to the laboratory colony Keele strain (Table S1). (C) Proportions of genes with differential transcript levels between the predominant molecular form at each location and the less abundant form. The numbers of genes with higher and lower transcript abundance are indicated by arrows pointing up and down, respectively (Tables S3, S4). (D) The proportions of genes with differential transcript abundance between the S and M molecular forms in each location. The numbers of genes with higher and lower transcript abundance are indicated by arrows pointing up and down, respectively. Equivalent differences in transcript abundance in the two locations are indicated in the overlapping section (Tables 1, S5). (E) Bars indicate the number of functional gene groups showing higher (upper bars) and lower transcript abundance (lower bars) (Tables S3–S5).

transcript abundance of genes that largely belonged to the stress-redox-mitochondrial functional group.

The higher transcript abundance of stress-related genes and the larger number of repressed genes in the less abundant form is likely to reflect general conditions of higher stress that might result from the environmental conditions or from inter-form competition. Studies in yeast have shown that stressful conditions can result in a

**Table 1.** Genes displaying consistent differential transcript abundance ($\geq$1.7-fold) between the *Anopheles gambiae* S and M molecular forms at both sampling locations (thus molecular form specific transcriptome patterns). Transcript abundance is indicated as the normalized intensity ratio in fold regulation. Minus values indicate the –fold lower transcript abundance in the experimental sample

| Expression levels specific of the molecular form | | | Ndom S vs M | Nson M vs S | Nson S vs M |
|---|---|---|---|---|---|
| Gene ID | Gene ID | Gene name | | | |
| *Immunity* | | | | | |
| ENSANGT00000008707 | AGAP011952-RA | caspase short class CASPS3 | −1361 | 1599 | −1599 |
| *Transport* | | | | | |
| ENSANGT00000010009 | AGAP006518-RA | Charged multivesicular body 1B chromatin modifying | 2513 | −1675 | 1675 |
| *Diverse* | | | | | |
| ENSANGT00000013925 | AGAP001215-RA | Tetratricopeptide repeat containing | −1343 | 1506 | −1506 |
| *Unknown* | | | | | |
| ENSANGT00000029116 | | Unknown | 1869 | −1365 | 1365 |
| ENSANGT00000025814 | | Unknown | 1852 | −1452 | 1452 |
| ENSANGT00000010799 | AGAP007620-RA | Unknown | 1703 | −1396 | 1396 |

twofold larger number of repressed genes; presumably, this response represents a strategy to protect critical functions. A reduced synthesis of irrelevant transcripts and their products may help to conserve energy while the organism tries to adapt to the suboptimal conditions (Gasch & Werner-Washburne, 2002). Similarly, our data suggest that the S molecular form in Nkolbisson, which represents only 17% of the population and showed a 1.7-fold higher number of repressed genes than did the M form, is in a disadvantageous situation with respect to its tolerance of the environment when compared to the M molecular form, with which it competes for resources.

In order to identify potential transcription signatures that are molecular form-specific and may underlie some of the consistent physiological differences between the two forms, we looked at genes that displayed a consistent differential transcript abundance between the two molecular forms in both geographical locations (Fig. 3D; Table 1). The fact that only six such genes were identified underscores the strong effect of environmental adaptation on the mosquitoes' physiology (Fig. 3D; Giger *et al.*, 2006). Genes that displayed higher transcript abundance in the M molecular form included a caspase (CASPS3), which is likely to be involved in apoptosis (Adrain *et al.*, 2004; Martin & Baehrecke, 2004), and a tetratrico-peptide repeat containing protein; this type of repeat has been found to act as a scaffold for a broad range of protein-protein interactions and is involved in the regulation of RNA synthesis or mitosis (Ben-Yehuda *et al.*, 2000). Genes that displayed high transcript abundance in the S molecular form included a charged multivesicular body chromatin modifying protein that is located in the nuclear matrix and plays a role in the formation of vesicle-filled endosomes that target proteins to the interior of lysosomes, affecting chromatin structure and cell cycle progression (Stauffer *et al.*, 2001). Another three genes with unknown functions (ENSANGT00000029116, ENSANGT00000025814, AGAP007620-RA) also showed an elevated transcript abundance in the S form. None of these six genes displaying differential transcript abundance could be linked to a potential mechanism related to the speciation process on the basis of its predicted function. It is possible that the speciation mechanisms act on mating behaviour and that the genes involved are expressed or repressed at the time of the male-female mating interaction, as has been seen for the morphs Cosmopolitan and Zimbabwe of *Drosophila melanogaster,* which are in an incipient speciation process (Michalak *et al.*, 2007). In spite of our observation, a study comparing gene transcript profiles between laboratory colony *A. gambiae* M and S forms at different life stages (fourth instar larvae, virgin females and gravid females) has shown 164 transcripts differently expressed between virgin females of the two molecular forms; amongst these

genes were several with putative implication in olfaction and mate recognition (Cassone *et al.*, 2008). Although transcriptome comparison was carried out between laboratory colonies, the transcript abundance patterns of a subset of genes were validated by RT-PCR on wild mosquitoes from Cameroon and Burkina Faso. The differences in results between Cassone' *et al.*'s study and ours are likely to be related to differences in the experimental design and the field sites from where mosquitoes were collected, as well as the largely different gene transcript abundance assay platforms. Cassone *et al.* (2008) used the Affymetrix GeneChip and we used an oligonucleotide glass slide array. Furthermore, the two platforms had been based on different *A. gambiae* genome annotations, and hence a large number of genes represented on one platform was lacking on the other. In our study, the proportions of the two molecular forms in each location were different, suggesting a strong and different effect of both environments on their physiology. This different effect is reflected in their transcriptomes, and is likely to be masking potential transcriptome signatures that are specific to each molecular form. By contrast, field mosquitoes used in Cassone *et al.*'s (2008) study were collected from different localities and breeding sites and pooled according to their molecular form: this procedure would result in the masking of the effect of environmental variation towards detection of specific transcriptomic attributes of each molecular form.

## Concluding remarks

Our study has shown a substantial divergence, at the transcriptome level, between laboratory colony and field mosquitoes, suggesting a significant impact of environmental conditions on the evolution of the mosquito transcriptome. Gene transcript abundance evolves at a much greater rate than do DNA sequences, by at least an order of magnitude. For instance, at least 10% of the nematode transcriptome is differentially expressed in organisms separated by 280 generations (Gibson, 2005). Mutations in a variety of different loci can affect transcript abundance, as gene regulation is affected by both *cis*- and *trans*-acting elements and transcription factors; furthermore, a single mutation in a single regulatory locus can affect the transcript abundance of dozens of target genes (Brem *et al.*, 2002; Rockman & Kruglyak, 2006; Wray, 2007).

Our comparison between the M and S molecular forms of *A. gambiae* from the Nkolondom and Nkolbisson locations has indicated that the similarity of transcript abundance is larger between the predominant forms from each location than between populations of the same molecular forms in the two locations, suggesting a strong effect of the environmental conditions on the mosquitoes'

physiology. The similarity in the transcript abundance profile between the dominant forms in the two locations is likely to reflect a better adaptation, and hence a less-stressed physiology. Differences in the ability of the mosquitoes to adapt to the environment and to utilize the available resources could result in 'competitive exclusion' between the two forms at each location, the outcome of which might be directly linked to the relative frequencies of each form at the time of colonization of the site.

## Experimental procedures

### Field sites and mosquito collection

*Anopheles gambiae* larvae and pupae were collected from several pools of water in two market gardening areas (Nkolondom and Nkolbisson) located in the outskirts of Yaoundé, Cameroon, and transferred to the insectary, where they were reared to the adult stage. The village of Nkolondom (3°58′20″N, 11°30′56″E) (A in Fig. 1) is about 9.5 km north-west of Yaoundé and outside the city limits. Nkolbisson (3°52′21″N, 11°27′15″E) (B in Fig. 1) is about 6.5 km west of the centre of the city, within an urbanized neighbourhood. Nkolondom overlooks the city at an elevation of 805 m, whereas Nkolbisson lies in a valley at 702 m. The sites are 10 km apart and are separated from one another by a steep hill with its peak at 1200 m (Fig. 1). They experience a typical four-season equatorial climate, with a mean annual rainfall of about 1500 mm and mean temperature of 24 °C. Although rains are recorded every month, the long dry season extends from late November to early March (with 10–30 mm rainfall/month), and the short dry season includes July and August (80–100 mm rainfall/month). The rainfall peak is in October (250–350 mm/month). Larval collections were conducted at both sites in November and December, 2004.

### Mosquito rearing and identification

Eggs of the *A. gambiae* Keele laboratory colony strain (a mixture of the M and S molecular forms) were brought to the insectary at the Organisation de Coordination pour la tutte contres les Endemies en Afrique Central (OCEAC) Research Station in Yaoundé, and larvae and adults were raised there for several generations under standard temperature and humidity conditions of 28 ± 1 °C, 70–80% relative humidity with a 12h:12h light-dark cycle. Larvae were fed on baby fish food (TetraMin Baby, Swell UK Ltd, UK) and adults were provided with 10% sugar solution *ad libitum*.

Fourth instar larvae and pupae were collected from natural breeding sites using dippers and transported to the laboratory where they were reared to adults in their own breeding site water, and maintained under temperature, humidity and feeding conditions identical to those of the laboratory colony mosquitoes. Emerging adults from field collections were readily identified on morphological grounds using reference keys (Gillies & De Meillon, 1968; Gillies & Coetzee, 1987), and only female *A. gambiae s.l.* specimens were included in the study. They were identified to species and molecular form using the PCR-restricted fragment length polymorphism method (Fanello *et al.*, 2002). One or two legs of each specimen were directly used as a DNA template source in the PCR reaction mixture.

In agreement with the known geographical distribution of species within the *A. gambiae* complex in this area (Wondji *et al.*,

2005; Ayala *et al.*, 2009; Simard *et al.*, 2009), only *A. gambiae s.s.* was present in our collections. Both the M and S molecular forms were found at both sites, and no hybrids were observed. In the more rural Nkolondom area, 90% (408/453) of the mosquitoes were of the S molecular form and 10% (45/453) of the M form, whereas in the more urbanized and polluted area of Nkolbisson, the M form was predominant (96/115 = 83%) over the S form (19/115 = 17%).

### Mosquito dissections and RNA extraction

Four-day-old sugar-fed females were used for gene transcript abundance analyses. After the mosquito was anaesthetized with $CO_2$, the head was removed to avoid eye pigment contamination, which can inhibit probe labelling reactions and the legs were dissected out for use in molecular identification (above). The remaining body was maintained in RNA later (Applied Biosystems/Ambion Inc., Austin, TX, USA) for subsequent RNA extraction using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA).

### Microarray-based transcription analysis

In each of the microarray–based transcription assays two pools of 20 mosquitoes were compared, except for the Nkolbisson S molecular form from which the pool was of 19 mosquitoes.

Fluorochrome-labelled cRNA probes were synthesized from 2–3 μg RNA extracted from the pools of mosquitoes using the Agilent Technologies low-input RNA labelling kit (Agilent Technologies, Palo Alto, CA, USA) according to the manufacturer's instructions and previously described methodology (Dong *et al.*, 2006). Probe quantity was estimated with a Beckman DU640 spectrophotometer (Beckman, Brea, CA, USA). Hybridizations were carried out on the previously described Agilent Technologies-based *A. gambiae* full genome glass slide microarray with the Agilent Technologies *in situ* hybridization kit according to the manufacturer's instructions and previously described methods (Dong *et al.*, 2006).

Microarray scanning was carried out with an Axon GenePix 4200AL scanner, and scan images were analysed with Genepix Pro 6.0 software (Axon Instruments, Union City, CA, USA). Images were analysed with Genepix 6.0 software to determine spot size, location and quality, and potentially confounding spots were manually removed from the analysis. The minimum signal intensity was set to 150 fluorescent units, and the signal-to-background ratio cut-off was set to 2.0 for both the Cy5 and Cy3 channels. For the transcriptomic comparison between laboratory colony and field mosquitoes, at least three biological replicates were performed for each experimental set. For the transcriptomic comparison between the M and S molecular forms, the three biological replicates were performed using three pools of mosquitoes of the predominant molecular form against one pool of mosquitoes of the less abundant form because of the low numbers of mosquitoes of the latter at each collection site. In all experiments, technical replicates were performed when the hybridization quality was considered insufficient based on signal to background ratio. The background-subtracted median fluorescent values were normalized according to a locally weighted scatterplot smoothing (LOWESS) normalization method, and Cy5/Cy3 ratios from replicate assays were subjected to *t*-tests with a 0.01 *P*-value using The Institute for Genomic Research (TIGR) Midas and Mev software (Dudoit *et al.*, 2003). Transcript abundance values for genes were included when significant *P*-values

were found amongst replicates of an experimental set. Transcript abundance ratios were averaged with the GEPAS microarray pre-processing software prior to logarithmic (base 2) transformation (Herrero *et al.*, 2003). All the transcript abundance values presented exhibited reproducible regulation trends (up- or down-regulation) in the replicate assays.

*Validation of microarray-based transcription data by real-time quantitative RT-PCR*

Microarray-assayed transcript abundance was further validated with quantitative RT-PCR for eight genes in the two sets of laboratory vs. field mosquito comparisons. RNA samples were reverse-transcribed using Superscript II (Invitrogen, Carlsbad, CA, USA) with random hexamers. Real-time quantification was performed using the QuantiTect SYBR Green PCR Kit (Qiagen) and ABI Detection System ABI Prism 7300 (Applied Biosystems, Foster City, CA, USA). All PCR reactions were performed in triplicate. The specificity of the PCR reactions was assessed by analysis of melting curves for each data point. The ribosomal protein S7 gene was used for normalization of cDNA templates. The gene names, IDs and primer sequences used are listed in Table S6. Microarray- and real-time quantitative PCR-generated data showed a high degree of correlation (Pearson correlation coefficient, $r = 0.96$; best-fit linear-regression, $R^2 = 0.93$; slope of the regression line, $m = 1.174$; Fig. 2; Table S7).

## Authors' contributions

Ruth Aguilar: performed experiments, analysed data and wrote the manuscript.
Frederic Simard: designed experiments and wrote the manuscript.
Colince Kamdem: performed experiments.
Tim Shields: analysed data.
Gregory E. Glass: analysed data.
Lindsey Garver: performed experiments and analysed data.
George Dimopoulos: coordinated the project, designed experiments, analysed data and wrote the manuscript.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: DOI 10.1111/j.1365-2583.2010.01031.x

**Table S1.** Genes displaying constitutively and significantly (≥1.7-fold difference) higher or lower transcript levels in field mosquitoes (Nkolondom-S and Nkolbisson-M mosquitoes), as compared to laboratory strain mosquitoes (Keele strain).

**Table S2.** Genes that showed differential transcript abundance (≥1.7-fold) between the *Anopheles gambiae* S and M molecular forms at both geographical locations (thus specific for the predominant form).

**Table S3.** Genes displaying differential transcript abundance (≥1.7-fold) between the *Anopheles gambiae* S and M molecular forms in Nkolondom.

**Table S4.** Genes displaying differential transcript abundance (≥1.7-fold) between the *Anopheles gambiae* S and M molecular forms at the Nkolbisson location.

**Table S5.** Genes that showed differential transcript abundance in each location for the same molecular form.

**Table S6.** PCR primers used for microarray data validation by real-time quantitative RT-PCR.

**Table S7.** Individual gene transcript abundance values (log$_2$ ratio) obtained by microarray and real-time RT-PCR to validate microarray gene transcript abundance data.

**Table S8.** Genes with similar transcript abundance in the field mosquitoes (S from Nkolondom and M from Nkolbisson) and in the Keele laboratory colony.

**Table S9.** Genes with similarly transcript abundance in the field S form mosquitoes from Nkolondom and M form mosquitoes from Nkolbisson and with differential transcript abundance when compared to the Keele laboratory colony.

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