Evolution of sexually-transferred steroids in *Anopheles* mosquitoes

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Abstract

Human malaria, transmitted by some *Anopheles* mosquitoes, remains a major public health problem. Unlike almost every other insect species, males of some *Anopheles* species produce and transfer steroid hormones to females during copulation to mediate reproductive changes. Steroids are consequently seen as a target for malaria vector control. However, a small fraction of mosquitoes has been studied, including malaria vector species only. Here, we analysed the evolution of sexually-transferred steroids and their effects across *Anopheles* by using a large set of mosquito species, including malaria vector and non-vector species. We show that male steroid production and transfer are specific to the *Cellia* subgenus, and are not interdependent with mating-induced phenotypes in females. Therefore, male steroid production, transfer and post-mating effects in females do not correlate with their ability to transmit human malaria, which overturns the suggestion from previous studies and suggests that manipulation of steroid-response pathways in the field does not represent an appropriate vector control strategy.

Anopheles mosquitoes are mostly known as they transmit malarial *Plasmodium* parasites that infect mammals. Among 472 species named in this genus, 41 have been classified as dominant vector species (DVS) of human malaria (*1-3*). The *Anopheles* genus is further subdivided into 8 subgenera of which 3 (*Anopheles*, *Cellia*, and *Nyssorhynchus*) contains all known DVS of human malaria (*3*). Despite a significant decrease of malaria incidence since 15 years due to vector control and improvement of chemoprevention, diagnostic testing and treatment, human malaria remains a world-wide burden with more than 200 million cases reported and an estimated 429 000 deaths in 2015 (*4*). With the increase of insecticide resistance in malaria mosquitoes (*4*, *5*), new vector control strategies to limit and possibly eliminate malaria transmission are being developed such as replacement of wild malaria-susceptible mosquito populations by resistant ones and/or decrease of vector populations by manipulating mosquito reproduction (*6*, *7*). Unlike vertebrates, it was considered that insect adult males do not produce significant amounts of steroid hormones until it was shown that males of *Anopheles gambiae* (*Cellia* subgenus), the main vector of human malaria in Africa, produce and transfer high quantities of 20-hydroxyecdysone (20E) to females during copulation (*8*). While ovarian steroid synthesis occurs in mosquito females upon blood meal triggering egg development (*8-13*), sexual transfer of steroids by males likely represents a nuptial gift that affects female reproduction in the malaria vector. Consistent with this, sexually-transferred steroids were shown to induce post-mating refractoriness and to stimulate oviposition in *An. gambiae* mated females (*14*). A report further suggested that production of steroids by male *Anopheles* mosquitoes, their transfer to females during mating and mating-induced phenotypes would be specific to dominant human malaria vectors, likely determining these mosquito species' ability to transmit malaria to humans (*15*, *16*). Steroids became as such a promising target to manipulate mosquito female reproduction with the aim to reduce malaria vector populations specifically. Consequently,

field use of 20E receptor agonists has been recently proposed to reduce malaria transmission (*17*). However, a limited number of *Anopheles* species have been studied. Indeed, male steroid production was assessed in 9 species that are all considered as DVS, and mating-induced phenotypes were analysed in 3 species only, 2 of which transfer steroids and one not. In the present work, we investigated the evolutionary history of male steroid production and the effect of its transfer during copulation on female reproductive traits using a large set of mosquito species.

We first measured steroid titers in whole sexually mature and virgin males from 19 different mosquito species. These mosquito species were selected within two *Culicidae* subfamilies, *Anophelinae* and *Culicinae*. Within the *Anophelinae* subfamily, 16 species distributed all over the world were chosen to cover 3 different subgenera (*i.e. Cellia, Nyssorhynchus* and *Anopheles*) of the *Anopheles* genus (Figure 1A; pictures of mosquitoes in Figure S1). In the *Culicinae* subfamily, *Aedes aegypti*, *Aedes albopictus* and *Culex pipiens* that are vectors of arboviruses were also analysed. As shown on Figure 1B (right panel), male 20E production occurs only within the *Cellia* subgenus (*Anophelinae*) and is absent in the *Culicinae*. Interestingly, *Anopheles quadriannulatus* whose males produce similar levels of steroids compared to *Anopheles stephensi* is not a malaria vector unlike *An. stephensi* and other *Cellia* species investigated in the present study. Indeed, its refractoriness or low susceptibility to the human malaria parasite has been experimentally determined (*18, 19*), confirming epidemiological data (*20*). Conversely, production of 20E was undetected in male mosquitoes from the two other *Anopheles* subgenera, *Anopheles* and *Nyssorhynchus* of which all tested members are registered as DVS and/or experimentally shown to be highly susceptible to *Plasmodium falciparum* (*21, 22*).

To uncover the evolutionary history of male steroid production in mosquitoes, we further consolidated the phylogenetic relationships of the species we analysed. To this aim, we used DNA sequences of partial coding gene regions of mitochondrial genes (*COI, COII, ND5 and CYTB*) and nuclear genes (*g6pd* and *white* as well as ribosomal subunits *18S* and *28S*) from the 19 mosquito species plus *Chagasia bathana* (*Anophelinae* subfamily, *Chagasia* genera) as outgroup for *Anopheles* mosquitoes (*23*). Phylogenetic analysis of the data set was performed by Bayesian inference (Figure 1B left panel) and by maximum likelihood (Figure S2). Phylogenetic relationships inferred from these analyses resulted in different topologies mainly at the subgenus level and with varying node support values. In both analyses, the members of each subgenus formed monophyletic groups and the branching orders within the subgenus *Cellia* was identical. Our results are in agreement with ones obtained by Sallum *et al.* (*24*) with the *Cellia* clade being the outgroup of the subgenera *Anopheles and Nyssorhynchus* in the Bayesian approach while in the maximum likelihood approach, *Nyssorhynchus* is the outgroup of *Anopheles* and *Cellia*, also in agreement with recently published phylogenies (*1, 25, 26*). From the Bayesian phylogenetic analysis and using fossil data, we obtained species divergence estimates, which are roughly conform to recently published phylogenies (*25, 27, 28*) (Table S1). Results indicate that the age of the last common ancestor of *Anopheles* genus is about 84.1 Ma (112.7-55.8, 95% confidence interval) and the most ancestral node within the *Cellia* subgenus is dated to 69.2 Ma (93.0-45.6, 95% confidence interval) (Figure 1B, Table S1). As males from all *Cellia* species tested so far have the ability to produce 20E, according to the parsimony law steroid production by mosquito males probably originated once in the early *Cellia* lineage, at about 84.1-69.2 Ma. (112.7-45.6, 95% confidence interval), *i.e.* during the late Cretaceous. Thus, steroid production by mosquito males is most probably a shared derived character from the last common ancestor of *Cellia* mosquitoes and represents as such a synapomorphy of this subgenus. *An. stephensi*

males transfer steroid upon mating to females (Figure S3) like in *An. gambiae* (*8*) and other *Cellia* species such as *Anopheles arabiensis* and *Anopheles dirus* (*15*), strongly suggesting that transfer of steroids to females during mating is part of the "male 20E production" synapomorphy of *Cellia* mosquitoes.

With the aim to get a deeper understanding of the effect(s) of sexually-transferred steroids in *Cellia* female mosquitoes, we investigated the influence of mating on two female reproductive traits which are known to be regulated by ovarian 20E. These traits are: i) detachment of ovarian follicles from the germarium in non-blood fed (NBF) females and ii) egg development in blood fed (BF) females. Indeed, steroid hormones trigger ovarian follicle detachment from the germarium in *Ae. aegypti* and *An. stephensi* (*9, 29*) on the one hand. On the other hand, ovarian steroids produced upon blood feeding stimulate vitellogenesis and egg development (*9, 11-13, 30*). We therefore analysed these two phenotypes in virgin and mated females from 12 *Anopheles* species (8 *Cellia*, 3 *Anopheles* and 1 *Nyssorhynchus*). As expected, mating induces the separation of the secondary ovarian follicle from the germarium in *An. stephensi* (Figure 2, see also confocal pictures Figure S4), a *Cellia* species whose males produce and transfer steroids during mating. Although to a lesser extent, mating induces as well the separation of the secondary follicle in *Anopheles minimus* and *Anopheles merus*, also from the *Cellia* subgenus. However, no secondary follicle detachment was observed after mating in *An. dirus*, another *Cellia* species, nor detachment of the third follicle for the *Cellia* species whose secondary follicle is already fully detached in virgin females (*Anopheles farauti*, *An. gambiae*, *An. arabiensis* and *An. quadriannulatus*). Among the species whose males do not produce steroids, mating also induces secondary follicle detachment in some species (*Anopheles atroparvus* and *Anopheles freeborni*), but not in others, even in species showing partial detachment of the secondary follicle in virgin females (*Anopheles*

quadrimaculatus and *Anopheles albimanus*). Similarly, mating increases the number of developed eggs in some mosquito species while not in others and this is not correlated to male steroid production (Figure 3 and Table 1). While it has been previously shown that mating can increase egg development in *An. gambiae* and *An. arabiensis* (*Cellia*, whose males produce steroids) (*15*) but not in *An. albimanus* (*Nyssorhynchus*, whose males do not produce steroids) (*15, 31*), experiments presented here and repeated with three independent cohorts of mosquitoes show that there is an effect of mating on egg development in *An. albimanus* but not in *An. gambiae* nor in *An. arabiensis*, regardless of the animal or human blood source used to feed mosquito females (Figure S5). It is likely that some variations can be observed between strains of a single *Anopheles* species as already described for some *Aedes* species (*32, 33*). It might also be possible that male steroids transferred upon mating benefit reproduction of *Cellia* females but only under certain environmental conditions as different ecological pressures such as nutrition can favour or not the maintenance and importance of nuptial gifts as shown for *Ae. aegypti* (*34-36*). Of note, across the species investigated, there is no correlation between the differences in mating-induced effects in females and the initial virgin females' status of secondary follicle detachment or ability to develop a high or low number of eggs after blood feeding. Moreover, among species within the *Cellia* subgenus, the occurrence or absence of mating-induced phenotypes in females are not linked to the different quantities of steroids produced by the cognate males (summarised in Table 1) and presumably transferred to females during mating as determined for *An. gambiae* and *An. stephensi*. Indeed, mating, most probably *via* male steroids, triggers both secondary follicle detachment and a rise in the number of developed eggs in *An. stephensi*, only secondary follicle detachment or increase in egg production in species such as *An. dirus* and *An. merus*, while steroid transferred by males do not induce at all these responses in species like *An. gambiae* and *An. arabiensis*, two species whose males transfer high amounts of steroids to females as

compared to *An. stephensi* and *An. dirus* (Figure S3, (*8, 15*)). Overall, our analysis demonstrates that post-mating responses increasing reproductive success in females indeed exist in *Cellia* species, some of which are likely mediated by male steroids. Importantly, they also occur in mosquito species whose males do not produce and transfer steroids such as in species belonging to the *Anopheles* and *Nyssorhynchus* subgenera (summarised in Table 1). Recently, male 20E has been shown to induce refractoriness to further mating in *An. gambiae* mosquitoes (*14*). Similarly to results obtained for the two post-mating responses analysed in this study, female monoandry (insemination by a single male) occurs in species whose males produce steroids but also in ones whose males do not (*37-40*). While few data are available for mosquitoes, it is well known that post-mating responses are quite conserved among insects. However, the rapid evolution of insect reproductive systems often results in species-specific genes and signalling pathways that ultimately trigger similar post-mating changes in different insect species (*41*). For instance, males transfer to females upon copulation Juvenile Hormone (JH) in *Ae. aegypti* mosquitoes and male accessory gland peptides such as Sex Peptide in *Drosophila* flies to trigger physiological and behavioural changes in mated females (*42, 43*). Likewise, males from *Cellia* species transfer steroid hormones while males from *Anopheles* and *Nyssorhynchus* species are likely to transfer other and not yet identified molecule(s) to achieve similar effects. As JH is transferred to female during mating in *Ae. aegypti* mosquitoes but also in the Lepidoptera *Heliothis virescens* (*44*), sexual transfer of steroid hormones in other mosquito subgenera, genera or even insect orders not yet tested cannot even be excluded.

A deep understanding of selective forces driving reproductive strategy diversity and their functional consequences are critical for designing strategies for management of insect pests. Analysing the evolution of steroid production by male mosquitoes from a large set of

mosquito species reveals that this physiological trait is a synapomorphy of the *Cellia* subgenus and importantly, that there is no correlation between the evolution of sexually-transferred 20E and malaria transmission to humans (summarised in Table 1). Consistent with this, while we show that male steroid production and subsequent transfer to females is likely to have evolved only once in the common ancestor of *Cellia* species, phylogenetic analyses on malaria mosquitoes support a convergent evolution with independent acquisitions of vectorial capacities in *Anopheles* mosquitoes (*45-47*). Also, a previous study has suggested that post-mating responses in *Anopheles* mosquitoes only exist in species whose males produce and transfer steroids to females shaping malaria vectorial capacities (*15*). Here we demonstrate that mating-induced phenotypes are variable among species and possibly even among strains or under different environmental conditions. These differences are independent of males' ability to produce and transfer steroids to females and not correlated to malaria vectorial capacity (summarised in Table 1). Apart from follicle detachment, increase in egg development and induction of refractoriness to mating, numerous other functions of steroids have been described in adult insects (*14, 15, 48-53*). Thus, sexually-transferred steroids could mediate different functions with more or less direct benefit for the female's reproduction in *Cellia* females, due to the rapid evolution of reproductive systems between species. Therefore, manipulating sexual-transfer of steroids in mosquitoes or using 20E receptor agonists in the field to manipulate *Anopheles* mosquitoes transmitting malaria to humans should be considered with caution in order to benefit malaria vector control strategies and also because it risks to affect other untargeted non-malaria vector species.

It still remains open as what were the evolutionary forces that have initially promoted the acquisition and radiation of this new, and presumably costly male steroid production and sexual gift to females in *Cellia* mosquitoes. It is striking that all tested species whose males

produce steroids and belong to the *Cellia* subgenus (Figure 1A) are absent from the "New World" but restricted to the "Old World". This is consistent with our contemporary geographical distribution mapping of nearly all mosquito species belonging to the *Cellia* subgenus (224 species) against the ones of *Anopheles* subgenus (184 species) (Figure 4). This suggests that the common ancestor of the *Cellia* subgenus originated in Gondwana, and then diverged - with the acquisition of male steroid production and transfer to female - after separation of South America and Africa, in agreement with previous observations (*3, 54*). This biogeographic calibration is consistent with divergence times obtained from our phylogenetic analysis placing the origin of steroid production by males of the *Cellia* subgenus around 84.1-69.2 Ma (112.7-45.6, 95% highest posterior density interval), *i.e.* after the separation of South America and Africa, which started at least 100 Ma ago with no land bridge for about 80 to 50 Ma (*55, 56*). At the late Cretaceous, two main paleogeological events that had impact on environmental conditions might have led to environmental pressures driving the evolution of steroid production and transfer by males in *Cellia* species: i) while Gondwana areas shared a common environmental history and were populated by the same plants and animals, the reconfiguration of oceans and lands as Gondwana broke up had profound global effects on environment but also on the specific environmental conditions in the newly formed continents with a divergence of plant and animal species (*57*); ii) the Cretaceous-Paleogene extinction event at 65.5 Ma, which timely correlates with the divergence time estimate for steroid production in *Cellia* males, has also been a dramatic world-wide event leading to disruption of ecosystems, with the disappearance of nearly all large vertebrates and many tropical non-vertebrate species (*58, 59*). Because such geographical isolation and environmental stresses are believed to drive traits contributing to animal species survival, it is likely that transfer of steroids by males to females have favoured *Cellia* species populations at this critical time.

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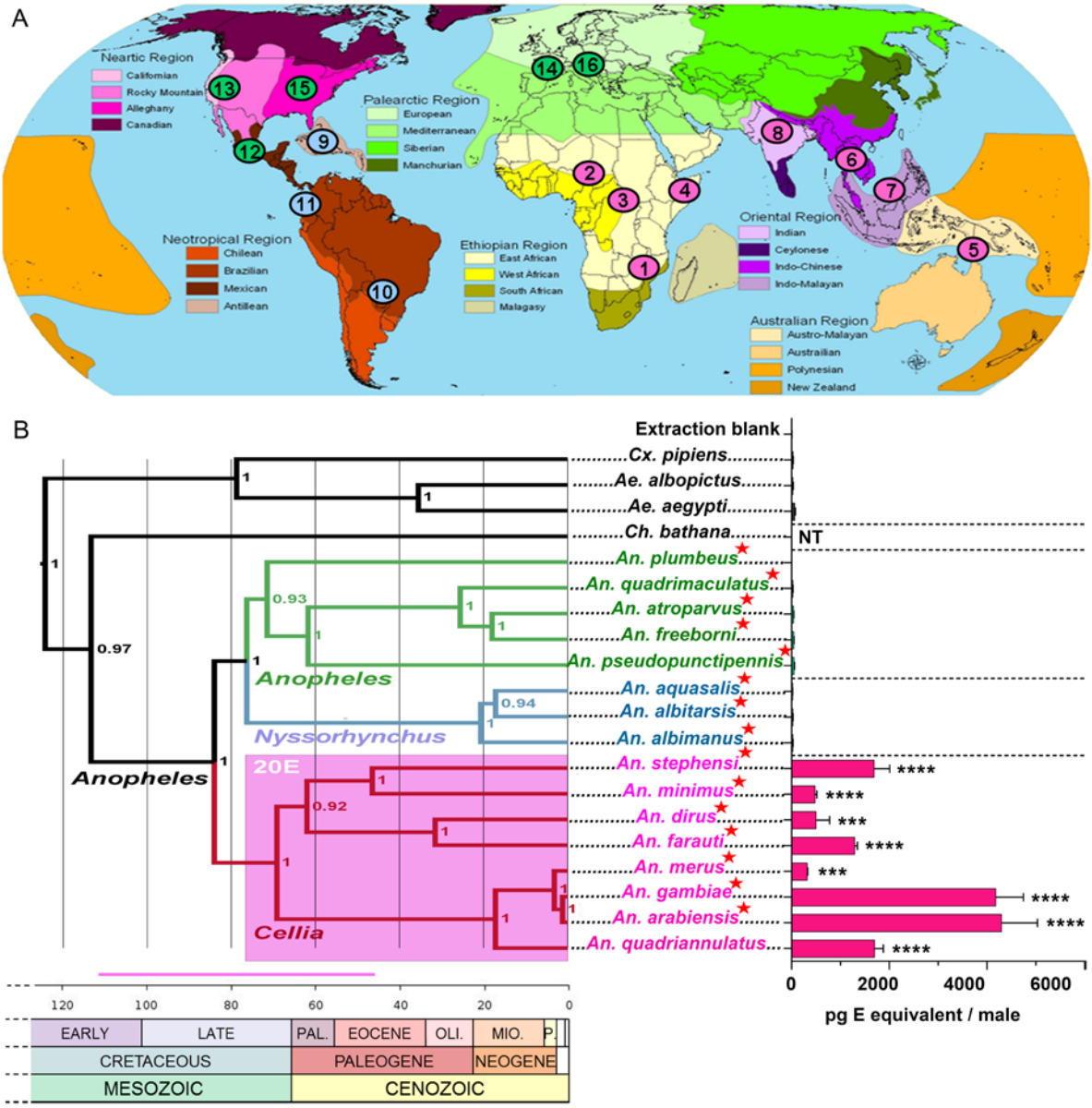

Fig. 1: Distribution, phylogenetic relationships of mosquitoes and steroid production in mosquito adult males. (A) Present geographical distribution of the 16 mosquito species (*Anopheles* genus) analysed in this study represented on a zoogeographical map (modified from The geographical distribution of animals, Eckert IV projections department of geosciences, Texas tech university). 1: *An. quadriannulatus*, 2: *An. arabiensis*, 3: *An. gambiae*, 4: *An. merus*, 5: *An. farauti*, 6: *An. dirus*, 7: *An. minimus*, 8: *An. stephensi*, 9: *An. albimanus*, 10: *An. albitarsis*, 11: *An. aquasalis*, 12: *An. pseudopunctipennis*, 13: *An. freeborni*, 14: *An. atroparvus*, 15: *An. quadrimaculatus*, 16: *An. plumbeus*. Pink: *Cellia*

subgenus, blue : *Nyssorhynchus* subgenus, green : *Anopheles* subgenus. (B) Bayesian phylogeny of 20 *Culicidae* species, 19 species tested for ecdysteroid male production plus *Chagasia bathana* (subfamily *Anophelinae,* genus *Chagasia*) used as outgroup for phylogenetic analyses. Dominant malaria human vectors are indicated by a red star. Time is represented in millions of years (Ma). Approximated node ages are also represented in Extended data 2. Bayesian node support values are presented on the right side of each node. Ecdysteroid titers in whole 5-day-old virgin males are represented on the right side of the tree. Results are expressed as mean +/- SEM in pg E equivalents per male. Results were subjected to statistical analysis using Kruskall-Wallis test for nonparametric data followed by Dunn's post-test (control group: Extraction blank). The indicated p values are those obtained with Dunn's test (***, p value < 0.001; ****, p value < 0.0001). NT: not tested. Predicted lineages with significant male 20E production are shaded pink on the tree. The pink horizontal bar represents the minimum/maximum 95% confidence interval (CI) estimated time in Ma for origin of male 20E production. The geological time scale is adapted from the Geological Society of America (http://www.geosociety.org/science/timescale/). The white coloured cases represent the quaternary period. PAL., Paleocene; OLI., Oligocene; MIO., Miocene; P., Pliocene.

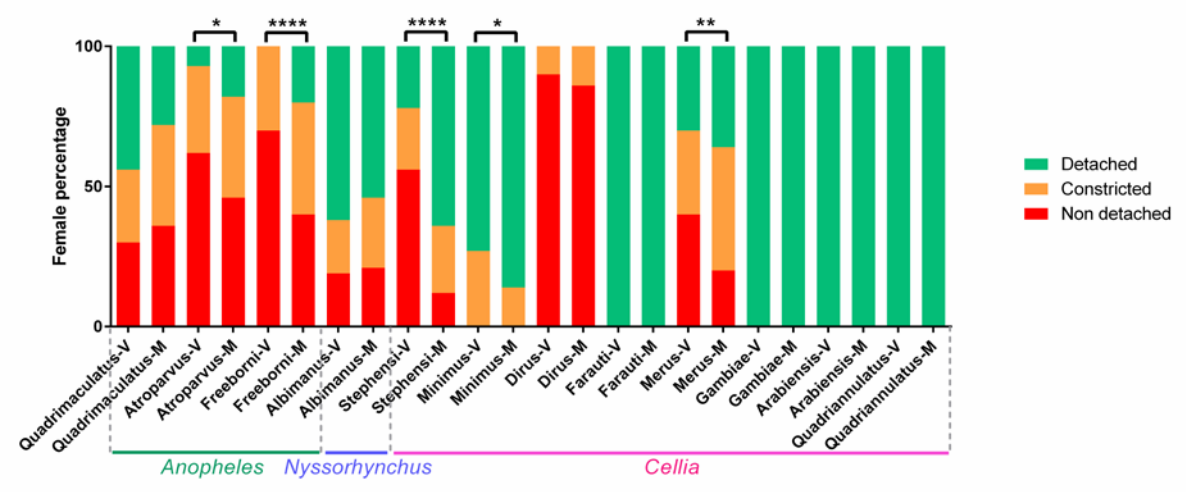

Fig. 2: Secondary follicle detachment from the germarium in virgin and mated non blood-fed females from 12 *Anopheles species*. Secondary follicle detachment from the germarium in ovarioles of virgin (V) and mated (M) females. Secondary follicles are either detached from the germarium (detached, green), in the progress of detachment (constricted, yellow) or non-detached yet (non-detached, red). The secondary follicle are significantly more detached in mated females compared to virgin females for *An. atroparvus* ($p=0.0226$), *An. freeborni* ($p<0.0001$), *An. stephensi* ($p<0.0001$), *An. minimus* ($p=0.0228$) and *An. merus* ($p=0.0072$).

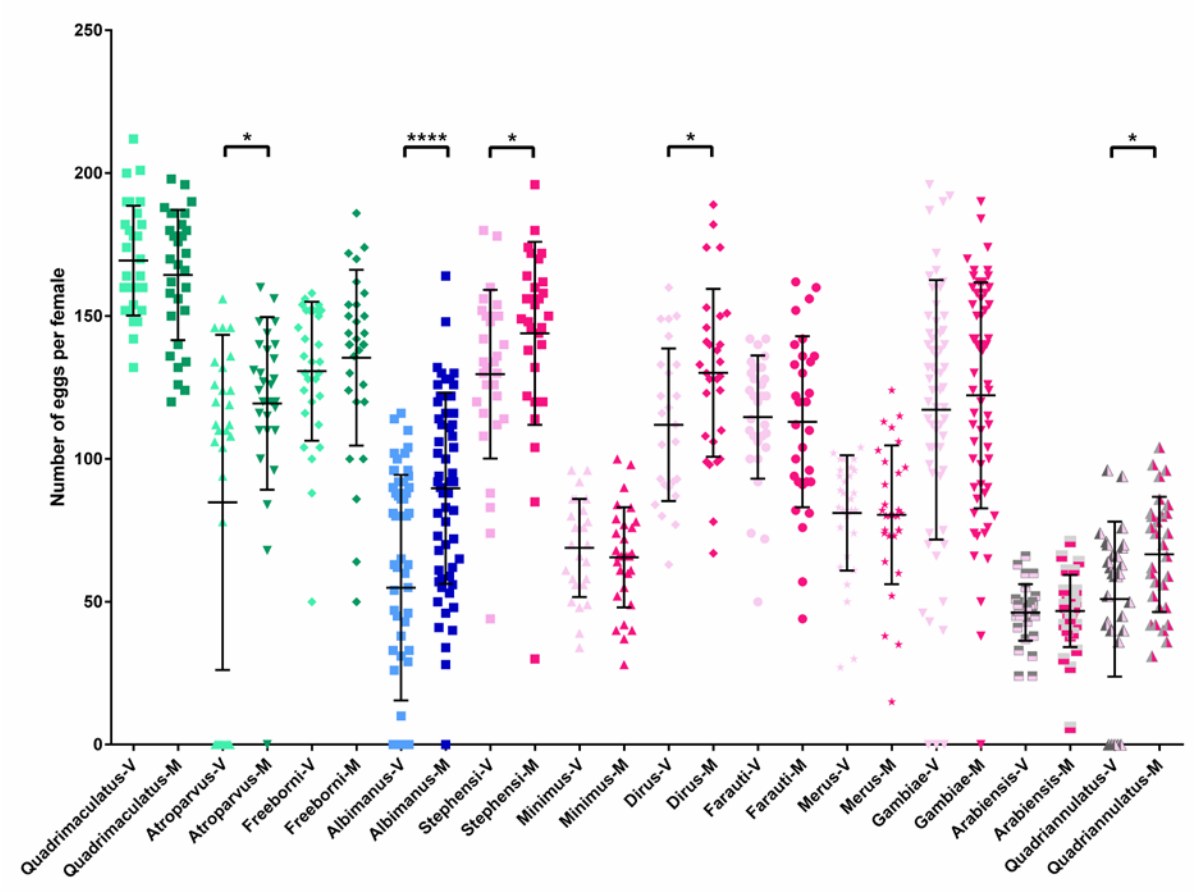

Fig. 3: Egg development in virgin and mated blood-fed females from 12 species of *Anopheles* mosquitoes. Total number of eggs in virgin (V, light colours) and mated (M, dark colours) females 48 hours after blood feeding. Green: *Anopheles* subgenus, blue: *Nyssorhynchus* subgenus, pink: *Cellia* subgenus. Females from *An. atroparvus* (Mann-Whitney U=295.5, p= 0.0214), *An. albimanus* (Mann-Whitney U= 941.5, p<0.0001), *An. stephensi* (Mann-Whitney U= 232.5, p= 0.0235), *An. dirus* (Mann-Whitney U= 298, p= 0.0240) and *An. quadriannulatus* (Mann-Whitney U= 309.5, p= 0.0373) develop significantly more eggs when they are mated.

Subgenus	Species	DVS	Male steroid production	Follicle detachment	Egg development
Anopheles	*An. quadrimaculatus*	-	-	-	-
	An. atroparvus	+	-	+	+
	An. freeborni	+	-	+	-
Nyssorhynchus	*An. albimanus*	+	-	-	+
Cellia	*An. stephensi*	+	++	+	+
	An. minimus	+	+	+	-
	An. dirus	+	+	-	+
	An. farauti	+	++	-	-
	An. merus	+	+	+	-
	An. gambiae	+	+++	-	-
	An. arabiensis	+	+++	-	-
	An. quadriannulatus	+	++	-	+

Table 1: Summary of mating effect on follicle detachment and egg development in regard to malaria vector status and male steroid production in *Anopheles* species. Dominant vector species (DVS) of human malaria are signalled by a +. For male steroid production, relative low titers are indicated by +, medium titers by ++, and high titers by +++. For follicle detachment and increase of egg development, - indicates no effect of mating and + indicates an effect of mating on these reproductive traits in females.

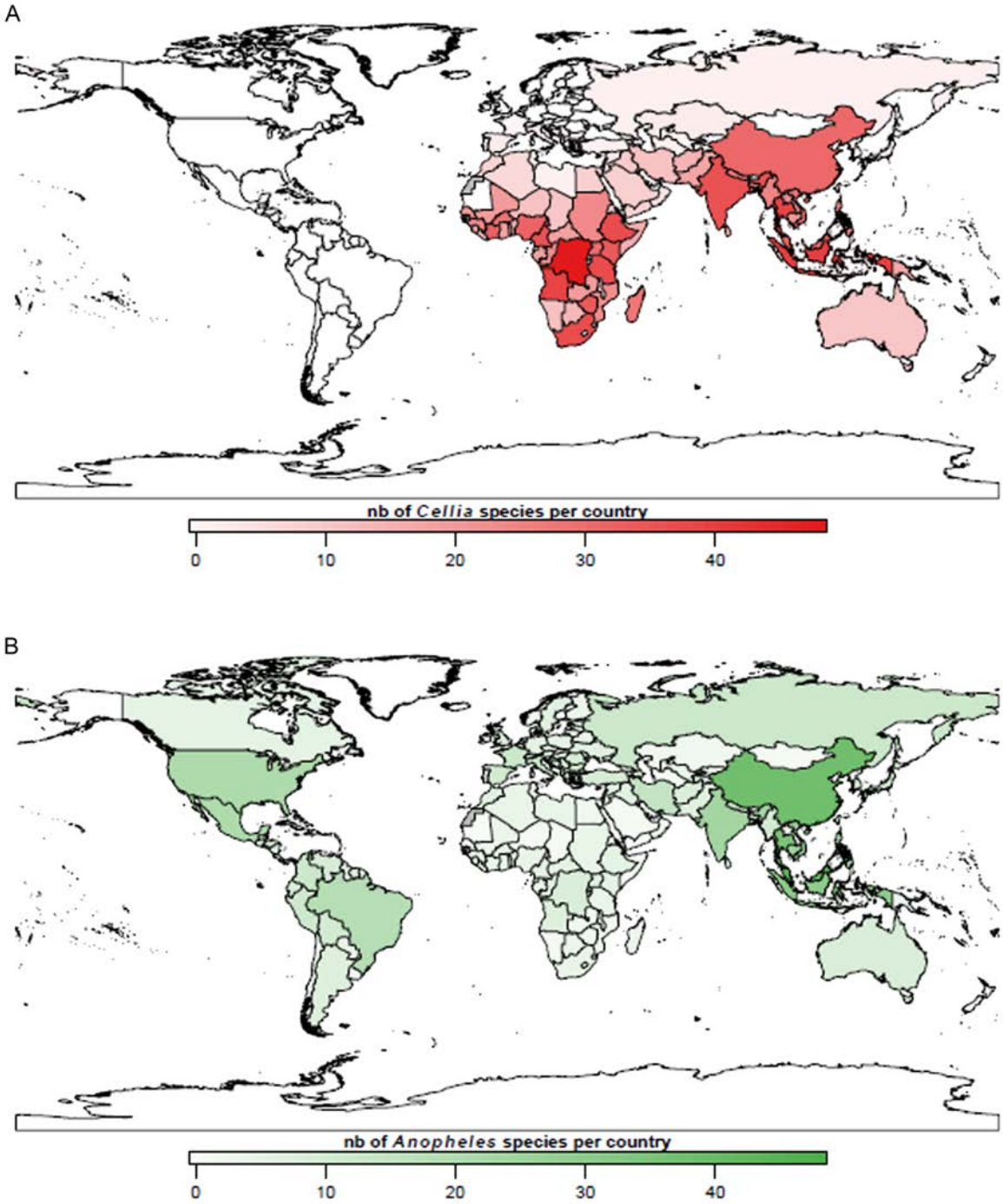

Fig. 4: Present geographic distribution of species belonging to *Cellia* and *Anopheles* subgenera (*Anopheles* genus). Total numbers of mosquito species belonging to the *Cellia* (a, red) and *Anopheles* (b, green) subgenera per country (sourced from the Walter Reed Biosystematics Unit, http://www.wrbu.org/) are represented on world maps created with R. Numbers (nb) of mosquito species per country are represented by a coloured gradient as depicted under each map. Grey colour means no data are available for the country.

Supplementary Materials:

Materials and Methods

Figures S1-S5

Tables S1-S5

References (*8, 24, 45, 60-77*)