Dual role of the *Anopheles coluzzii* Venus Kinase Receptor in both larval growth and immunity

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Vector-borne diseases and especially malaria are responsible for more than half million deaths annually. The increase of insecticide resistance in wild populations of *Anopheles* malaria vectors emphasises the need for novel vector control strategies as well as for identifying novel vector targets. Venus kinase receptors (VKRs) constitute a Receptor Tyrosine Kinase (RTK) family only found in invertebrates. In this study we functionally characterized *Anopheles* VKR in the Gambiae complex member, *Anopheles coluzzii*. Results showed that *Anopheles* VKR can be activated by L-amino acids, with L-arginine as the most potent agonist. VKR was not required for the fecundity of *A. coluzzii*, in contrast to reports from other insects, but VKR function is required in both *Anopheles* males and females for development of larval progeny. *Anopheles* VKR function is also required for protection against infection by *Plasmodium* parasites, thus identifying a novel linkage between reproduction and immunity in *Anopheles*. The insect specificity of VKRs as well as the essential function for reproduction and immunity suggest that *Anopheles* VKR could be a potentially druggable target for novel vector control strategies.
Transcripts of vkr were found to be abundant in larval forms and in female ovaries of the platyhelminth S. mansoni and of several insects like Tribolium castaneum, Apis mellifera, Anopheles gambiense. They were found in the ovaries of Aedes aegypti and more recently in the gonads of the desert locust, Schistocerca gregaria. Functional studies using RNAi-mediated genes silencing showed that VRKs control reproduction, and especially the production of eggs in S. mansoni as well as in the mosquito vector A. aegypti in which VKR was demonstrated to be a receptor for the ovary ecdysteroidogenic hormone (OEH). VRKs were also shown to control testis development and spermatogenesis in S. mansoni male parasites. Unlike in A. aegypti and S. mansoni, VKR does not seem to be essential for reproduction in S. gregaria, since silencing of SgVKR did not affect fecundity or fertility.

Here we performed the functional characterization of Anopheles VKR in Anopheles coluzzii, a member of the Gambiae species complex of major malaria vectors. We cloned the VKR gene from an A. coluzzii laboratory strain and expressed the protein in stage VI Xenopus oocytes. These cells are blocked in the first period of meiosis (metaphase 1) but activation of RTK can induce further kinase signalling cascades and Akt and MAPK phosphorylation can result in meiosis resumption and Germinal Vesicle BreakDown (GVBD). In this heterologous system, we demonstrated the TK activity of A. coluzzii VKR and identified L-Arginine as its most potent activating ligand. In addition to expression in the mosquito gonads, we also found that vkr transcripts were expressed in Anopheles haemocytes, which are immune-competent cells. Functional studies using RNAi-mediated gene silencing revealed a dual role for the A. coluzzii VKR in both the reproduction of this insect, as well as immunity against infection with malaria parasites.

Materials and Methods

Cloning of Anopheles VKR. The VKR of A. gambiae M (now named A. coluzzii) was identified by screening the available expressed sequence tag (EST) and genomic databases (Flybase: [http://flybase.org/]) with the protein sequence of SmVKR1 from Schistosoma mansoni using the TBLASTN program. The complete cDNA sequence of A. coluzzii VKR was determined by 5’ and 3’ RACE amplification (GeneRacer Kit, Invitrogen) according to the manufacturer’s instructions. Fragments encompassing the complete coding region of VKR were obtained by PCR on adult mosquito cDNA using Advantage 2 Polymerase mix (Clontech Laboratories, Inc.) and the following primer sequences: AgVKRf1 (5’-GCCTGCGGTTGGACATCTGGGC-3’) and AgVKRr1 (5’-GCTGCGGTTGGACATCTGGGCC-3’). PCR products were purified from agarose gels using the extraction kit Wizard SV Gel and PCR clean-up system (Promega) and inserted into pCR2.1-TOPO (Invitrogen). Selected clones of full length VKR were sequenced by GATC Biotech. The A. coluzzii VKR nucleotide sequence was previously deposited in Genbank under the name AgVKR with the accession number EU878397.1.

VKR constructs and site-directed mutagenesis. AgVKR cDNA sequence was subcloned into the mammalian expression vector pCDNA3.1-V5-His (Invitrogen) (VKRWT) by an in frame insertion using EcoRI and NotI sites. Site-directed mutagenesis was used to change the Serine 505 to Alanine in the VFT domain of VKR (VKRS505A) using the QuikChange Site–Directed Mutagenesis Kit (Stratagene). The 5’-CTGGGCTCCTGGGCAGTTGAGCCATTGC-3’ mutated sequence and its reverse complement were used as primers to mutate Serine 505 into Alanine (mutated residues are in lowercase italics).

Expression of recombinant VKR in Xenopus oocytes. cRNAs encoding VKRWT or VKRS505A proteins were synthesized in vitro from plasmids previously linearised by Pmel enzyme using the T7 Message Machine Kit (ambion, USA). cRNA transcribed from 1 µg of each linearised plasmid was precipitated with 2,5 M LiCl, washed in 70% ethanol, resuspended in 20 µl diethylpyrocarbonate (DEPC)-treated water, then quantified by spectrophotometry. Finally, 1 µg of cRNA was analysed on a denaturing agarose gel. Gel staining with 10 µg/ml ethidium bromide allowed confirmation of the correct size of cRNA and verification of the absence of abortive transcripts. 60 ng of cDNA preparations (1 mg/ml) were microinjected in Xenopus laevis oocytes in the equatorial region according to the protocol previously described18 and incubated for 18h at 19 °C in ND96 medium (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM Hepes pH 7.4 supplemented with 50 µg/ml streptomycin/penicillin, 225 µg/ml sodium pyruvate, 30 µg/ml trypsin inhibitor) supplemented or not with L-amino acids and/or kinase inhibitors in order to evaluate VKR kinase activation and induction of germinal vesicle breakdown (GVBD) in oocytes, a process easily detectable by the appearance of a white spot at the centre of the animal pole of the oocyte. Expression of VKR proteins in oocytes was confirmed by immunoprecipitation of membrane extracts according to the procedure described previously18 using anti-V5 antibodies (1:100, Invitrogen). Immune complexes were analysed by Western blotting using anti-V5 (1:50 000) or PY20 (1:10 000, antiphosphotyrosine, BD Biosciences) antibodies and the advanced ECL detection system (Amersham Biosciences).

Nucleotide and protein sequence analyses. Sequence analyses were performed using the LASERGENE package (DNAStar, Madison, WI, USA). The AgVKR cDNA was used to determine the genomic structure using BLAST analysis (http://flybase.org/blasti/) on FlyBase data bank. The exon–intron boundaries were slightly modified by eye. The signal peptide, the VFTM and TK domains were delimited by SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/), InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/) and BLAST structural analysis algorithms. Motif research was made using ELM server (Eukaryotic Linear Motif: http://elm.eu.org). Pair-wise mannered alignments were generated with Clustal W program (MEGA4).

RNA interference. The N’gousso mosquito colony, initiated in Cameroon in 2006, then implemented in 2007 in the CEnter for Production and Infection of Anopheles (CEPIA) at Pasteur Institute, was used for functional tests. The colony, previously described as A. gambiae M molecular form, is A. coluzzii in the current nomenclature. Gene specific transcripts of Anopheles VKR and GFP (dsRNA control) were generated from A.
Anopheles cDNA or GFP-containing plasmids by PCR using T7 promoter-tagged primer sequences (T7-VKR-F/T7-VKR-R and T7-GFP-F/T7-GFP-R respectively; Table S2). PCR products were used as templates for in vitro dsRNA synthesis using the MEGAscript RNAi Kit (Invitrogen). 500 ng of dsRNA were injected into the thorax of ice-anesthetized 1–2 days old females or 4 days old males A. coluzzii using a nanoinjector (Nanoject II; Drummond Scientific). Four days after the dsRNA treatment, knockdown efficiency of the Anopheles VKR gene was checked using total RNA from pools of five injected mosquitoes. Using 1 μg of total RNA, reverse transcription followed by quantitative PCR was performed with Anopheles VKR specific primers (VKR-q-F and VKR-q-R; Table S2). The same samples were also used to measure the expression level of the CYP307A and CYP314A, both involved in the biosynthesis of the 20-Hydroxyecdysone. Ribosomal gene rpS7 was used as an internal calibrator for normalisation. Specific primers for transcript quantification of CYP307A (CYP307-q-F and CYP307-q-R), CYP314A (CYP314-q-F and CYP314-q-R) and of the internal calibrator rpS7 (rpS7-q-F and rpS7-q-R) are listed in Table S2. Analysis of the expression of transcripts relative to rpS7 was performed according to the 2−ΔΔCt method. Difference in delatCt distribution between dsGFP and dsVKR was statistically tested using Wilcoxon signed rank non-parametric test.

Plasmodium Infection and oocyst detection. Females Anopheles previously injected with dsGFP (control group) or dsVKR were fed on mice infected with P. berghei strain PbGFPCON20, which constitutively expresses green fluorescent protein (GFP) and produces mature gametocytes. After the infectious blood meal, unfed mosquitoes were removed and fully engorged females were maintained at 21 °C and 70% relative humidity on 10% sucrose.

Mosquito midguts were dissected at 8 days post-infection, and the number of oocysts (the Plasmodium parasite stage located in the mosquito midgut) was counted by fluorescence microscopy. A mosquito was considered infected if at least one oocyst is seen in its midgut. Prevalence of infection is defined as the proportion of infected mosquitoes among the total dissected mosquitoes. For each gene-silencing experiment at least 25 surviving mosquitoes were analyzed for oocyst detection. For statistical analysis, differences in infection prevalence were tested using Chi Square. Following independent statistical tests for each replicate, and when the direction of change of each independent replicate was concordant, the p-values from independent tests of significance were statistically combined using the meta-analytical approach of Fisher.

Anopheles female ovary size and structure and number of developed oocytes. 4 days post dsRNA injection (dsGFP or dsVKR), Anopheles females were allowed to feed on naïve mouse for inducing vitellogenesis and eggs production. Dissection of individual female was performed at 48 h post-blood meal to collect their ovaries. The ovary size was measured under a light microscope (20× objective), where pictures were taken for each female ovaries from control dsGFP and dsVKR groups. A similar experiment was made, but the pictures of the ovary pairs were used to count the number of developed oocytes in each ovary pair. Differences in ovary sizes or in the number of developed oocytes between the two groups were tested.

Effect of VKR on fecundity and on progeny larval growth. Females (mothers) injection. Injections of dsGFP or dsVKR were performed in 1–2 days old virgin A. coluzzii females of. 3 days post injection the injected females were put in contact with 1-week old non-injected males A. coluzzii in a 1:3 ratio (1 male for 3 females). 6 days post-injection these females were allowed to take a blood meal on naïve mouse and were maintained at 25 °C, 75% humidity. Egg laying was assessed for individual females and for pools of 40 females. For the latter case, 72 h post-blood feeding, the number of laid eggs was counted and reported to the number of females (40 females mothers) as a proportion and the difference of this proportion between dsGFP and dsVKR groups was tested using Chi Square. For the eggs laid from the individual females, the number of eggs was counted individually and differences between the two groups were tested using non-parametric Wilcoxon Mann-Whitney.

For the larval growth, 72 h post-feeding the eggs are collected from the two groups of females (dsGFP and dsVKR), counted, put in water pans and stored at 26 °C for larval growth. The number of emerging adults from each pan (dsGFP or dsVKR) was counted and reported to the number of initial laid eggs, as a proportion. The difference of this proportion between dsGFP and dsVKR groups was tested using Chi Square.

Males (fathers) injection. Injections of dsGFP or dsVKR were performed on 4 days old virgin A. coluzzii males. In order to let them release their stock of spermatozoids acquired during their larval development, and to allow a renewal of spermatozoids under VKRkd background, the 2 groups of males were allowed to mate with a first group of virgin females, 2 days post-injection in a 1:3 ratio (1 male for 3 females). Then, 3 days later, the 2 groups of injected males were all collected and put in contact with a new batch of virgin females for 2–3 days (still in a 1:3 ratio). These second batch of virgin females mated with dsGFP or dsVKR males were allowed to feed on naïve mouse and were maintained at 25 °C, 75% humidity. As described above, eggs were collected 72 h post-feeding, put in water and allowed to hatch and become adult. As for the injected females, the proportion of emerging adults was compared between dsGFP or dsVKR groups using Chi Square.

Haemocyte collection from adult Anopheles females. Haemocytes were collected by perfusion from 20 Anopheles mosquito females as described in. Basically, the penultimate abdominal segment of each mosquito was cut and intrathoracic perfusion with an anticoagulant buffer (60% Schneider’s medium, 10% PBS, 30% citrate buffer [98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, 41 mM citrate]) was performed. Using a Hamilton syringe system. ~10 μl of perfused haemocoele was collected from each perfused mosquito on tubes stuck into ice. The collected haemolymph from the 20 mosquitoes was pooled and centrifuged at 1,500 rpm for 5 min at 4 °C in order to collect the haemocytes. The buffer was removed and TRIzol reagent (Invitrogen) was added to resuspend the haemocytes and performed a total RNA extraction.
Ethical and regulatory considerations. This study was conducted in strict accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals of the European Union (European Directive 2010/63/UE) and the French Government. All procedures were approved by the Hygiene and Security Commission of the Institut Pasteur (protocol CHSCT N°13.313). The experiments on *Xenopus laevis* were carried out in accordance with the principles of the European Community Council recommendations (86/609/EEC). The protocols were approved by the "Comité d’Ethique en Expérimentation Animale, Hauts de France" (CEEA, 07/2010). All methods were performed in accordance with the relevant guidelines and regulations.

Results

Cloning of *A. coluzzii* VKR. The reference genome assembly of *A. gambiae* was screened with a TBLASTN program to identify sequences related to the *S. mansoni* VKR sequence (SmVKR1). One EST sequence was identified as AGAP009158. The vkr gene is composed of six exons and five introns and is located on chromosome 3 R (Fig. 1A). The corresponding *A. coluzzii* gene (ACOM027165) was shown to display the same genomic organization. Using classic and RACE-PCR strategies, we cloned the complete cDNA sequence of VKR expressed by mosquitoes, which contains 5527 bp with a C terminal poly A tail. The sequence was submitted to Genbank under the name AgVKR (accession number EU878397).

Comparative sequence analyses of *Anopheles* VKR with other insect VKR proteins. Amino-acid sequences of the VFT and TK domains of *Anopheles* VKR were aligned with sequences of VKR from two other mosquitoes, *Aedes aegypti* (AaVKR) and *Culex pipiens quinquefasciatus* (CqVKR) and from the honeybee *Apis mellifera* (AmVKR) (Fig. 1B,C). The multiple alignment showed that the *Anopheles* VKR sequence is more closely related to those of mosquitoes (*Aedes* and *Culex*) (65% identity) than to bees (*Apis*) (45% identity) in the VFT domain. Sequence analysis of *Anopheles* VKR confirmed the typical structure of its VFT domain formed by two lobes and three linkers that confer flexibility for the open-close conformation of the VFT domain (Fig. 1B). Our previous studies have shown that SmVKR1 and AmVKR possess a partially conserved sequence consensus for amino-acid binding and this is also the case for *Anopheles* VKR.

The TK domain of *Anopheles* VKR is also more similar to other mosquito (92%) than to bee VKRs (75%) (Fig. 1C). Like the other VKRs, *Anopheles* VKR possesses the eleven subdomains present in all protein kinases and the characteristic motifs essential for tyrosine kinase activity. The GXGXXG motif in subdomain I is responsible for ATP binding and the VAVXXE sequence (Subdomains II and III) is required for ATP stabilization. In subdomain VIb, the HRDXAXRC sequence is implicated in the phosphotransfer on tyrosine residues. The DFG motif essential for the binding of Mg\(^{2+}\) is present in domain VII together with the two tyrosine...
residues, which are the site of receptor autophosphorylation in several RTKs including insulin receptors. Finally the PVRWMAPE sequence responsible for the conformation domain stabilization is present in subdomain VIII.

\[ L-\text{Amino Acids (AA) activate } Anopheles \text{ VKR through its VFT domain.} \]

We have previously shown that schistosome SmVKR could be expressed efficiently in *Xenopus* oocytes following injection of *in vitro* transcribed and capped messenger RNA\(^1\)\(^2\)\(^,\)\(^3\),\(^4\) and that several VKRs are effectively activated by L-AA\(^9\). The presence of the signature for L-AA binding in *Anopheles* VKR suggested that it could also be activated by these ligands.

It has been shown previously that expression and activation of VKR kinase induced germinal vesicle breakdown (GVBD) in *Xenopus* oocytes\(^1\)\(^2\). In this work, *Xenopus* oocytes were injected with cRNA encoding wild-type *Anopheles* VKR and we measured the capacity of the 20 classical L-AA to activate the VKR kinase and to induce GVBD. Results shown in Fig. 2A indicate that 7 L-AA (L-Arg, L-Ser, L-Ala, L-Glu, L-Thr, L-Gly and L-Cys) can induce GVBD and that L-Arg is the most potent agonist, already active at 1 \(\mu\)M whereas L-Cys is the least active, with a 1 mM concentration required (Fig. 2A). We also tested D-arginine and L-canavanine, which is structurally related to L-arginine, but not the presence of the inhibitor AG1024. The autophosphorylating activity of the mutant VKR\(^{505A}\) is strongly affected.

**Figure 2.** Functional characterization of *Anopheles* VKR in *Xenopus* oocytes. (A) Minimal L-AA concentrations to induce GVBD in *Xenopus* oocytes expressing *Anopheles* VKR (from 1 \(\mu\)M to 1 mM). Arginine is the most efficient L-AA to induce VKR activation. Mutation of Ser\(^{505}\) present inside the ligand binding pocket strongly decreases the capacity of VKR to be activated by L-AA. (B) Effect of various tyrosine kinase inhibitors on VKR activity induced by arginine binding evaluated by the percentage of GVBD in oocytes expressing VKR. AG1024, AG538 and HNMPA (IR inhibitors) and AG1478 (IR and EGFR inhibitor) totally inhibit *Anopheles* VKR activation at 1 \(\mu\)M whereas SU11274 (Met receptor inhibitor) has no effect at this concentration. (C) Native *Anopheles* VKR\(^{WT}\) or mutant VKR\(^{505A}\) expressed in oocytes were immunoprecipitated from membrane extracts of oocytes using anti-V5 antibodies and revealed by Western Blot using anti-V5 and anti-PY antibodies for detecting tyrosine phosphorylation. VKR\(^{WT}\) is found to be phosphorylated in the presence of the ligand arginine but not in the presence of the inhibitor AG1024. The autophosphorylating activity of the mutant VKR\(^{505A}\) is strongly affected.

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*Anopheles* VKR possesses the strictly conserved serine residue (S\(^{505}\)) already shown to be involved in the binding of the carboxylic group of \(\alpha\)-AA in VFT modules\(^23\),\(^24\) and previously demonstrated to be required for the binding of L-AA by schistosome VKR\(^13\). Similar experiments of VKR activation by L-AA were performed using a mutant version of *Anopheles* VKR in which the S\(^{505}\) was replaced by an alanine residue. Results showed that L-AA were no more able to trigger GVBD in *Xenopus* oocytes expressing the mutant VKR\(^{505A}\), except L-Arg, for which a 1 mM concentration was in this case necessary to induce GVBD (Fig. 2A), indicating the importance of S\(^{505}\) for ligand binding and activation of the *Anopheles* VKR. Western blot results (Fig. 2C) confirmed the expression of recombinant V5-tagged VKR at the oocyte membranes and the tyrosine phosphorylation of VKR in the presence of L-Arg. The inactive mutant VKR\(^{505A}\) is no more highly phosphorylated in the presence of L-Arg, and this indicates that a functional VFT domain is required for *Anopheles* VKR activation by L-AA.

**Anopheles** VKR is sensitive to insulin receptor kinase (IR) inhibitors. We further tested the sensitivity of the TK domain of *Anopheles* VKR to different tyrosine kinase inhibitors and evaluated the capacity of the tyrphostins AG1024, AG538, HNMPA (three IR inhibitors), AG1418 (IR/EGFR inhibitor) and SU11274 (Met inhibitor) to inhibit oocyte GVBD induced by L-Arg. At 1 \(\mu\)M, AG1024, AG538, AG1418, and HNMPA inhibited totally GVBD, whereas SU11274 had no effect (Fig. 2B). This result confirmed the similarity of the TK domain of VKRs with IR receptors\(^8\). Western blot analysis (Fig. 2C) confirmed the absence of tyrosine phosphorylation of L-Arg-bound VKR in the presence of 1 \(\mu\)M AG1024.
VKR silencing did not affect ovary structure and fecundity in *A. coluzzii*. VKR transcripts have been already shown to be abundant in *Anopheles* gonads. We therefore assessed whether VKR could play a role in *Anopheles* reproduction. To this aim, RNAi-mediated gene silencing was performed in two groups of two day-old virgin *Anopheles* females by intra-thoracic injection of dsRNA. One control group was injected with dsGFP and the other group with dsVKR. At three days post-injection, the females were allowed to mate with 7 day-old virgin males and were blood fed at day 6 post-injection.

Comparing the size and the structure of ovaries at 48 h post-blood feeding, we could not find any statistically significant difference between dsGFP and dsVKR females (Fig. 3A). We also counted the number of developed oocytes in the ovary pair at 48 h post-blood feeding and we did not find a statistical difference between the two groups of females (Fig. 3B). To strengthen this observation, we checked the female fecundity in both dsGFP and dsVKR groups by counting the number of laid eggs from both pool of females and from individual female. From the pool of females, results from four independent experiments presented in Fig. 3C showed no difference in the number of laid eggs/female between dsGFP and dsVKR females. Similarly, looking at the number of eggs from individual females did not show statistical difference between dsGFP and dsVKR groups (Fig. 3D). This indicates that VKR is not required for *A. coluzzii* fecundity.

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VKR is required for *Anopheles* larval development. As VKR silencing in females did not affect their fecundity (Fig. 3), we further assessed the capacity of laid eggs to develop from larvae to adults. Silencing was performed in the same experimental conditions as above in two groups of *Anopheles* females (dsGFP and dsVKR). Eggs from each group were collected at 72 h post-blood feeding and placed in water. As no difference was found...
for the hatching rate between eggs collected from dsGFP and dsVKR female mothers (Supplementary Fig. S1),
we then assessed the efficiency of these eggs to develop from larvae to adult stages. Three independent biological
experiments were performed which showed a statistically significant reduction (19%, p = 0.044) of the proportion
of emerging adults reported to the number of eggs put in water in VKR-silenced females (Fig. 4A).
As VKRs were previously considered to have functions in schistosome spermatogenesis12, we also assessed the
role of VKR in offspring of males. dsRNA injections were performed in two groups of 4-day old Anopheles
males (dsGFP and dsVKR), assuming VKR might be required for a good quality of spermatozoids. Therefore in order to
test VKR function in male gonads, spermatozoid renewal should occur under dsVKR backgrounds. To this aim,
two days post-dsRNA injection, the 2 groups of males were allowed to mate with a first group of virgin females
in order to make them releasing their stock of spermatozoids acquired during their development. After mating
with this first group of virgin females, the 2 groups of males (dsGFP and dsVKR) were put with a new group of
virgin females during 3 days. This second group of mated females were blood fed in order to collect their eggs
72 h post-feeding. As it was observed in VKR-silenced females, we did not see any difference in the number of
laid eggs from females mated with dsGFP or with dsVKR males (Supplementary Table S1). However, the develop-
ment of these eggs from larvae to adult stage was strongly affected in the group of eggs issued from VKR-silenced
fathers (p = 1e-05), since we observed more than 70% decreased survival in this larval group (Fig. 4B). This
stronger phenotype observed when VKR is silenced in males as compared to females might be due to a more
efficient silencing of vkr transcripts observed in males (Supplementary Fig. S3).
We then tried to perform a similar experiment in which mating occurred between males and females both
silenced for Anopheles VKR. However, when both partners were injected, we found out that the efficiency of mat-
ing, even in the dsGFP controls, was very low (with number of laid eggs lower than 10), and we could not pursue
the experiment.

**VKR is required for Anopheles immunity against Plasmodium infection.** In addition to expres-
sion in gonads, VKR transcripts were also detected in the rest of the body9. Haemocytes and the fat body have

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**Figure 4.** VKR is required in larval growth. VKR silencing either in female (A) or in male (B) mosquitoes
affects the larval development of their progenies, considering the proportion of emerging adults reported to
the number of eggs put in water. Stars (*) and (*** ) represent Chi2 test p-value < 0.05 and p-value < 0.001
respectively.
been involved in insect immunity, but VKR function in these cells and organ was not already examined. The demonstration that haemocytes perfused from adult *A. coluzzii* females express VKR transcripts (Supplementary Fig. S2) allowed us to hypothesize that VKR could also be involved in *Anopheles* immunity. As a tyrosine kinase receptor, VKR is linked to different kinase pathways and possibly in *Anopheles* to the MAP Kinase and the JNK kinase, which are involved in the anti-*Plasmodium* response.

To test a potential immune role for VKR, two groups of *A. coluzzii* females were injected with dsGFP (control) and dsVKR and 4 days post-injection, these two groups of females were infected with the rodent malaria parasite *Plasmodium berghei*. The midguts from the two groups of infected mosquitoes were dissected 8 days post-infection to assess their infection status. The results from three independent experiments showed an increase of infection prevalence in VKR-silenced background as compared to the dsGFP control group (Fig. 5). However no effect was observed on infection intensity (parasite load in infected midguts). These results reveal a novel function related to immunity for an insect VKR, in addition to its role in reproduction or in our case, in larval growth.

**VKR might be involved in the biosynthesis of 20E in *Anopheles* females.** Then we focused our interest on particular steroid hormones, named ecdysteroids, which, in insects, play a major role during growth, development and reproduction. The active form of the hormone is the 20-hydroxyecdysone (20E), which coordinates major developmental transitions. We assessed whether VKR could be linked to the biosynthesis pathway of the 20E looking at the expression level of two P450 enzymes, CYP307A1 and CYP314A1, that are both involved in the biosynthesis of 20E in *Anopheles*. We found that CYP314A1 expression level is reduced only in females silenced for VKR, but not in males (Supplementary Fig. S4). However CYP307A1 expression was not statistically affected by VKR silencing in both males and females.

**Discussion**
In this study, we report the functional characterization of a member of the VKR family in the mosquito vector *A. coluzzii*. This RTK is encoded by a single gene located on the 3R chromosome and sequence alignment with other insect VKRs revealed a high level of conservation of its functional domains. *Anopheles* VKR is, as the majority of the other VKR proteins, activated following the binding of arginine to the VFT extracellular domain and the structure of its TK domain is very similar to that of insulin receptors, an important characteristic of VKR molecules in terms of downstream kinase signalling. VKRs were first discovered in the helminth parasite *Schistosoma mansoni*, and they have been particularly studied in this organism for their function in reproduction. In this helminth, VKR is involved in larval growth processes but it is mainly required for the production of eggs by female worms. The function of VKR in egg maturation was then confirmed in the mosquito vector *A. aegypti*. In *Schistocerca gregaria*, VKR knockdown had significant effects on ovarian ecdysteroid levels and on the size of oocytes during the vitellogenic stage but it could not be concluded that VKR was essential for reproduction, since silencing did not affect fecundity or fertility in *S. gregaria*. Therefore, it was important to analyse the function
of these novel receptors in Anopheles in order to understand its importance in the biology and the physiology of this mosquito vector and to estimate the pertinence of such molecules, which are absent from the human host, as potential new vector targets.

The functional characterization of Anopheles VKR has been performed using RNAi-mediated gene silencing assays in A. coluzzii. Our results showed that VKR displayed no function in Anopheles fecundity, as its silencing near affected the structure of the ovaries, nor the number of laid eggs per female (Fig. 3A–C and Supplementary Table S1). Nevertheless, decreasing the expression of the gene either in females (mothers) or in males (fathers) significantly affected the development of their larval progeny until the adult stage and with a stronger effect in male progeny. The weaker phenotype observed in female mothers as compared to males might be due to the difference in the silencing efficiency of vkr transcripts between females and males (Supplementary Fig. S3). Finally, we found out that VKR expressed in circulating haemocytes could play a protective role against Plasmodium, since dsVKR females displayed an increase of Plasmodium infection prevalence as compared to the control group (Fig. 5). The participation of VKR in the anti-Plasmodium phenotype still requires further work to identify the pathway(s) and the downstream immune factor(s), which are linked or regulated by VKR. However we could not exclude that VKR might be linked to the MAP Kinase and the JNK kinase, which are involved in the anti-Plasmodium response (model, Fig. 6). This dual role of VKR in both larval growth and immunity is reminiscent of the role of the dorsal regulatory cassette in Drosophila. Dorsal controls dorsal-ventral patterning during embryonic development, but during the adult stage the Dorsal regulatory mechanism is reused to regulate completely different genes involved in immunity.[29,30]

We then assessed whether VKR could be linked to the biosynthesis pathway of the 20E looking at the expression level of two P450 enzymes, CYP307A1 and CYP314A1, that are both involved in the biosynthesis of 20E in Anopheles.[28] As we found that only CYP314A1 expression level is affected in females silenced for VKR, but not in males (Supplementary Fig. S4), we could not exclude that VKR probably acts through another mechanism in Anopheles. For instance, it has been demonstrated that in insect ovaries, a phosphorylated form of ecdysteroid (ecdysteroid 22-phosphate), which is physiologically inactive, could serve as a “reservoir” in the ovaries that supplies active free ecdysteroids during embryonic development triggered by a phosphatase.[11,12] The formation of the phosphorylated ecdysteroid (ecdysteroid 22-P) requires the activity of an ecdysteroid kinase. As a kinase receptor, we could hypothesize that VKR would act upstream of this ecdysteroid kinase, or would belong to the kinase pathway that leads to produce the ecdysteroid 22-P (Fig. 6). Hence depleting VKR in A. coluzzii would impair the ecdysteroid kinase pathway and reduce the amount of ecdysteroid 22-P. Therefore in VKR silencing background, the reservoir of active free ecdysteroids further required during the larval growth might be reduced and could significantly impair the larval development.

The evolutionary history and function of VKRs might be different between the various organisms in which they have been found. In the case of two phylogenetically close organisms, Aedes and Anopheles mosquitoes, we did not observe the same function between Aedes VKR[14] and Anopheles VKR, especially for the reproductive function. Aedes VKR displays an important function in fecundity, while Anopheles VKR does not. This might also be due to different ecological niches and different pressures acting on VKR genes. Nevertheless, as it was not checked in the study of AeVKR,[14] we could not exclude that the receptor also plays a role in males and in immu-

 Altogether and consistent with the importance of its biological effects (Fig. 6), Anopheles VKR appears, through its dual role and the absence of ortholog in vertebrates, as an interesting novel insect target for controlling mosquito populations.
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Author Contributions
N.G., E.B.F., A.P., N.M.Z., K.C., C.G., K.D.V. and C.M. conceived and designed the experiments. N.G., E.B.F., A.P., N.M.Z., K.C., C.G. and C.M. performed experiments and analyzed the data. N.G., K.D.V, C.D. and C.M. designed the plan, wrote and discussed the manuscript.
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