Insect Biochemistry and Molecular Biology 142 (2022) 103720

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Beyond the eye: Kynurenine pathway impairment causes midgut homeostasis dysfunction and survival and reproductive costs in blood-feeding mosquitoes

Vanessa Bottino-Rojas [a,1], Igor Ferreira-Almeida [b,1], Rodrigo D. Nunes [b,2], Xuechun Feng [c], Thai Binh Pham [a], Adam Kelsey [a], Rebeca Carballar-Lejarazú [a], Valentino Gantz [c], Pedro L. Oliveira [b,d,**], Anthony A. James [a,e,*]

[a] Department of Microbiology & Molecular Genetics, University of California, Irvine, CA, USA
[b] Instituto de Bioquímica Médica Leopoldo de Meis, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil
[c] Section of Cell and Developmental Biology, University of California San Diego, La Jolla, CA, USA
[d] Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular, Rio de Janeiro, RJ, Brazil
[e] Department of Molecular Biology & Biochemistry, University of California, Irvine, CA, USA

ARTICLE INFO

Keywords:
Tryptophan metabolism
Transgenesis
Kynurenine hydroxylase/monooxygenase

ABSTRACT

Insect ommochrome biosynthesis pathways metabolize tryptophan to generate eye-color pigments and wild-type alleles of pathway genes are useful phenotypic markers in transgenesis studies. Pleiotropic effects of mutations in some genes exert a load on both survival and reproductive success in blood-feeding species. Here, we investigated the challenges imposed on mosquitoes by the increase of tryptophan metabolites resulting from blood meal digestion and the impact of disruptions of the ommochrome biosynthesis pathway. Female mosquitoes with spontaneous and induced mutations in the orthologs of the genes encoding kynurenine hydroxylase in *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* exhibited impaired survival and reproductive phenotypes that varied in type and severity among the species. A compromised midgut permeability barrier function was also observed in *An. stephensi*. Surprisingly, mutant mosquitoes displayed an increase in microbiota compared to controls that was not accompanied by a general induction of immune genes. Antibiotic treatment rescued some deleterious traits implicating a role for the kynurenine pathway (KP) in midgut homeostasis. Supplemental xanthurenic acid, a KP end-product, rescued lethality and limited microbiota proliferation in *Ae. aegypti*. These data implicate the KP in the regulation of the host/microbiota interface. These pleiotropic effects on mosquito physiology are important in the development of genetic strategies targeting vector mosquitoes.

1. Introduction

Approximately half of the world's population is at risk for a mosquito-borne pathogen and disease with the most vulnerable populations being in the tropical and subtropical regions (WHO, 2017). Controlling mosquito populations to reduce human exposure to infected vectors remains the most effective method to limit these diseases. However, increased chemical applications for vector control have led to the emergence and spread of insecticide resistance in mosquito populations (Wilson et al., 2020). Furthermore, pathogen persistence and resurgence, as well as the resilience and adaptability of their vectors, continue to motivate the search for novel evidence-based solutions to prevent transmission (Shaw and Catteruccia, 2019; Wilson et al., 2020).

Blood-feeding behavior (hematophagy) has evolved independently

Abbreviations: Ae, Aedes; An, Anopheles; Cx, Culex; KP, kynurenine pathway; Kh, kynurenine hydroxylase; kh^{w}, kynurenine hydroxylase-white gene; Trp, tryptophan; Kyn, kynurenine; 3-HK, 3-hydroxykynurenine; XA, xanthurenic acid.

* Corresponding author. Department of Microbiology & Molecular Genetics, University of California, Irvine, CA, USA.
** Corresponding author. Instituto de Bioquímica Médica Leopoldo de Meis, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil.
E-mail addresses: pedro@bioqmed.ufrj.br (P.L. Oliveira), aajames@uci.edu (A.A. James).
[1] These authors contributed equally to this work.
[2] Present address: Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD, USA.

https://doi.org/10.1016/j.ibmb.2022.103720
Received 24 November 2021; Received in revised form 3 January 2022; Accepted 4 January 2022
Available online 6 January 2022

in multiple insect taxa as an effective means to acquire nutrient resources for reproduction and energy (Rivera-Pérez et al., 2017; Waage, 1979). As this process is essential to some species reproduction, its impairment presents a potential target for population control. The essentiality of hematophagy also provides an evolutionary route for direct transmission of human pathogens, including the mosquito-borne dengue, Zika and West Nile viruses, malaria-causing *Plasmodium* parasites, and the worms that cause lymphatic filariasis. Specific adaptations of these insects for hematophagy include unique modes of cell signaling, immunity and metabolism as well as genes adapted to facilitate blood feeding and digestion. One feature of hematophagy is the highly-biased amino acid composition of the diet, as proteins comprise 85% of blood dry-weight. This hyperproteic fuel stimulates midgut microbiota proliferation and results in a significant metabolic shift after feeding (Bottino-Rojas et al., 2015; Sterkel et al., 2017). Not surprising, 30–50% of the genes in females show modulated (up- or down-regulated) expression levels following a blood meal (Bonizzoni et al., 2011; Marinotti et al., 2006).

The catabolism of the amino acid tryptophan is well-studied in invertebrates because its main oxidative branch, the kynurenine pathway (KP), provides substrates for synthesis of ommochrome eye pigments that have long been used as genetic markers in the development of transformation technologies (Jasinskiene et al., 1998; Coates et al., 1998; Gantz et al., 2015; Figon and Casas 2019; Adolfi et al., 2020). Several of the KP intermediates are biologically-active metabolites (Fig. 1A). The KP starts with the conversion of tryptophan (Trp) to N-formylkynurenine catalyzed by tryptophan-2,3-dioxygenase (TDO), followed by transformation to kynurenine (Kyn) either through spontaneous hydrolysis or by the enzyme, kynurenine formamidase (KFase), and is further metabolized to 3-hydroxykynurenine (3-HK) by kynurenine hydroxylase (KH, also referred to as 3-monoxygenase, KMO). Vertebrate studies associate aging and neurodegenerative disorders with upregulation of Trp-Kyn metabolism (Savitz, 2020). The central KP branch in most insect species ends with the synthesis of the free-radical generator, 3-HK, the initial precursor for ommochrome synthesis. Inhibition of this main branch of Trp degradation prolongs life span in eye-color mutant strains of the vinegar fly, *Drosophila melanogaster* (Oxenkrug, 2010; Oxenkrug et al., 2011). Production of xanthurenic acid (XA) from 3-HK in mosquitoes is proposed to protect the organism from accumulation of 3-HK (Han et al., 2007; Lima et al., 2012). Large amounts of XA are accumulated in the midgut of the yellow fever vector, *Aedes aegypti*, where it has a proposed role as an endogenous antioxidant and in the control of midgut epithelial homeostasis (Lima et al., 2012).

Strains carrying mutations of KP genes are the basis of valuable *in vivo* models of insect genetic modification. In the diverse orders in which they have been well-studied (including flies, moths, bees and beetles), mutations at several different loci are known to cause easily-scored eye-color phenotypes (Figon and Casas, 2019; Summers et al., 1982). However, knowledge of the functions of the kynurenines in non-drosophilid insects remains largely restricted to their role as precursors for the ommochrome pathway. Groundbreaking transformation technologies in *Ae. aegypti* benefited from the existence of a strain carrying a spontaneous mutation that produced a recessive white-eye phenotype easily detectable in homozygous mutant mosquitoes (Bhalla, 1968; Cornel et al., 1997; Han et al., 2003). Remarkably, the phenotype could be rescued when complemented with a wild-type copy of the *D. melanogaster cinnabar* (*cn*) gene, thus definitively establishing orthology of the mosquito and vinegar fly genes (Cornel et al., 1997). The mosquito gene, named *white* when it was first discovered, was later renamed *kynurenine hydroxylase*^{white} (*kh*^{w}) to distinguish it from the family of similarly-named genes that encode ABC transporters in insects (Bhalla, 1968; Dermauw and Van Leeuwen, 2014; Jasinskiene et al., 1998). A wild-type *D. melanogaster cn*$^{+}$ allele was used as a dominant marker to rescue the *Ae. aegypti kh*^{W} white-eye phenotype in the first reliable transposon-based transgenesis systems in mosquitoes (Jasinskiene et al., 1998; Coates et al., 1998). Although used successfully in these studies, there were data indicating that homozygous mutant *kh*^{w} mosquitoes had lower fitness than wild-type strains (Coates et al., 1998; Jasinskiene et al., 1998; Kokoza et al., 2001). More recently, novel genome-editing tools such as transcriptional activator-like effector nucleases (TALENs) and CRISPR/Cas9 also targeted *kh*^{w} and orthologous genes as proofs-of-principle for mutagenesis in mosquitoes (Aryan et al., 2013; Basu et al., 2015; Feng et al., 2021a; Gantz et al., 2015; Li et al., 2017; Liu et al., 2019; Yamamoto et al., 2018). Furthermore, site-specific integration of a gene-drive construct carrying antimalarial effector genes into the *kh*^{w} locus of the Indo-Pakistan malaria vector, *Anopheles stephensi*, was accompanied by reduced fitness in the white-eyed, homozygous mutant females (Gantz et al., 2015). A subsequent laboratory cage trial study showed that the reproductive cost of disruption of both copies of the *kh*^{w} target gene significantly impacted the stability of the intended genetic modification in the population, with cage populations going to extinction or accumulating drive-resistant mutations (Pham et al., 2019).

Here, we investigate fitness parameters of respective homozygous null mutant mosquitoes carrying the *Ae. aegypti kh*^{w} spontaneous mutant allele or mutations in the orthologous genes of *An. stephensi* and the southern house mosquito, *Culex quinquefasciatus*, created by target site-specific deletions following Cas9-mediated, non-homologous end joining (NHEJ) (Feng et al., 2021a; Gantz and Bier, 2015; Pham et al., 2019). Our results reveal that the KP is an endogenous mediator of host-microbiota symbiosis, as mutant mosquitoes showed a bloodmeal-dependent decrease of survival and lifespan, together with increased midgut tissue permeability and reduced reproductive performance. Moreover, we emphasize the importance of kynurenine research, the complex role of the KP in the physiology of disease-vector mosquitoes, and implications of using *kh*^{w} and orthologous mutant strains as recipients or products of transgenesis and gene-drive studies.

2. Methods

2.1. Mosquito strains, rearing and feeding

The Red-Eye and *kynurenine hydroxylase-white* (*kh*^{W}, recessive white-eye mutant phenotype) *Aedes aegypti* strains were maintained at the Universidade Federal do Rio de Janeiro, Brazil. The *kh*^{W} strain (Bhalla, 1968) carries a natural spontaneous deletion of 162 nucleotides (nt) in the *kh/KMO* gene (AAEL008879) that results in a loss-of-function (null) allele (Han et al., 2003). The Red-Eye strain was used as a control group and has wild-type functioning of the tryptophan pathway (Lima et al., 2012). The mosquitoes were maintained at 28 °C and 80% relative humidity and 12-h light/dark cycle. Larvae were fed with powdered dog chow (Purina), and adults were provided with 10% sucrose solution *ad libitum*. To evaluate oviposition, previously blood-fed and fully engorged *Ae. aegypti* females were transferred to individual cages (10 × 15 cm height) and were fed with 10% sucrose solution *ad libitum* and allowed to lay eggs on a wet piece of filter paper for 7 days after the blood meal. For *Ae. aegypti* antibiotic treatment, 3-day-old adult mosquitoes were transferred to a previously-sanitized (75% ethanol) cage and provided with a sterile 5% sucrose solution containing a 0.1% antibiotic mix (10 U/ml penicillin, 10 μg/m, streptomycin, 12 μg/ml ampicillin and 15 μg/ml gentamicin). This solution was provided on a sterilized cotton pad and replaced every day for three days before each blood meal. Rabbit heparinized blood was provided through a glass feeding apparatus (Garcia et al., 1975), supplemented with 6 mM XA (Sigma-Aldrich, St. Louis, MO, USA) or the same volume of added vehicle for controls. Unless otherwise stated, insects were adult females between 5 and 8 days after eclosion.

A colony of *An. stephensi* (Jiang et al., 2014) bred at the University of California, Irvine (UCI) for over 15 years was used in the experiments. The strain used, 10.2.18 *white*, is homozygous for a mutant allele of the orthologous *kh*^{W} gene. Gene amplification and sequencing confirmed a Cas9-mediated NHEJ in-frame mutation that results in deletion of a

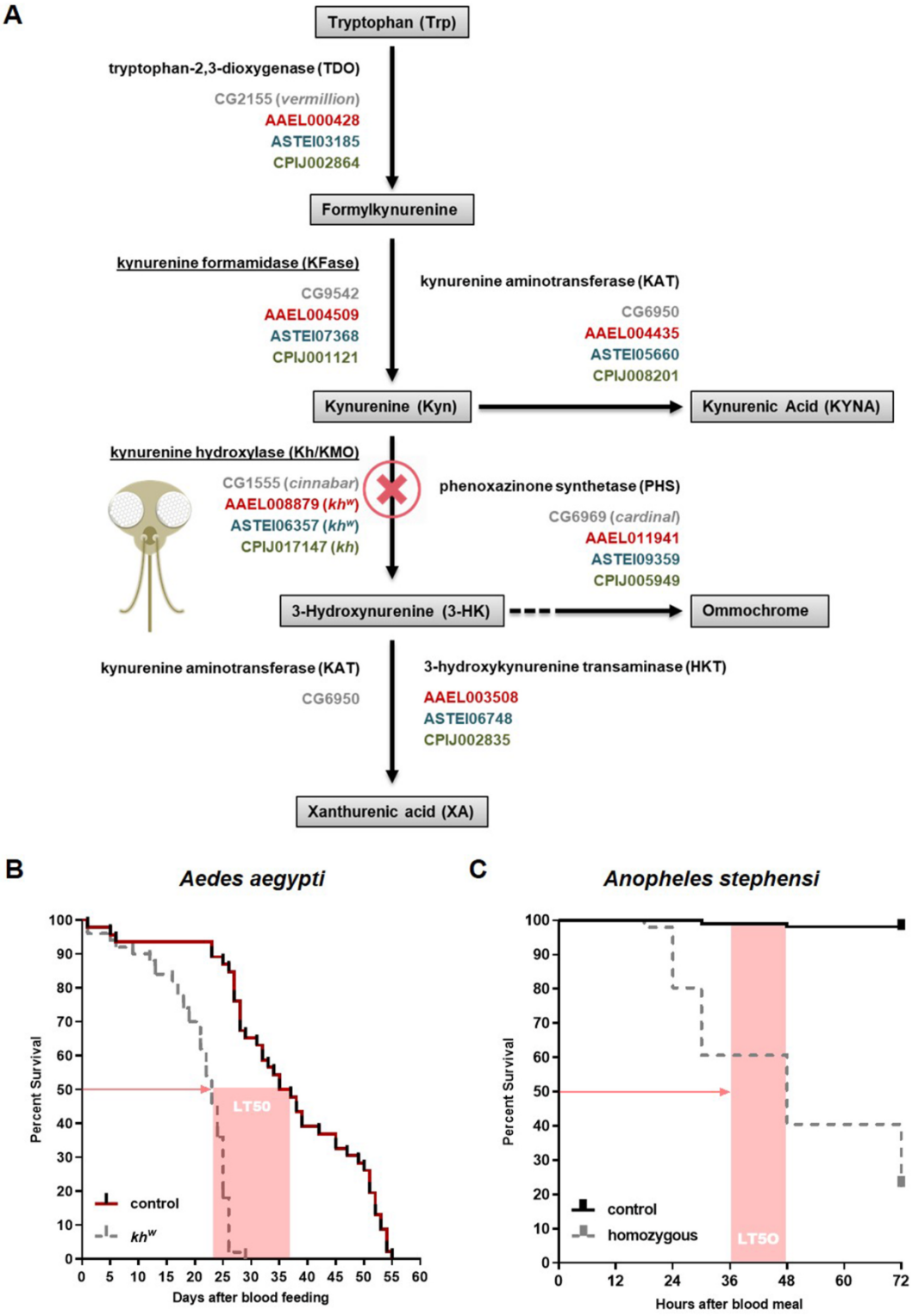

Fig. 1. Homozygous *kh* mutations impose a fitness cost following a blood meal. (A) Schematic representation of the kynurenine pathway (KP). KP metabolites are essential for formation of the ommochromes, biological pigments required for wild-type eye color in mosquitoes and are listed with abbreviations in the shaded boxes. Enzyme names and abbreviations are listed to the left or above the arrows indicating their role in the metabolic pathway. The ortholog gene IDs are provided for each of the encoded enzymes: *Drosophila melanogaster* (CG), *Aedes aegypti* (AAEL), *Anopheles stephensi* (ASTEI) and *Culex quinquefasciatus* (CPIJ). Gene names of kynurenine hydroxylase (3-monoxygenase) (Kh/KMO) mutations are provided. Fitness-impaired, homozygous mutant *kh* mosquitoes have disruption of the KP pathway caused by the loss of function of the KH enzyme and resulting in 'white-eye' phenotype (red 'X'). **(B)** Homozygous kh^{w} (kh^{w-}/kh^{w-}) mutations decrease the survival of adult female *Aedes aegypti* following a blood meal. In this experiment, control (wild-type) and kh^{w} mutant mosquitoes were compared. LT50 (red arrow) represents the median Lethal Time (time until death) for each experimental group after initial blood feeding and the pink bar indicates the differential in the two groups. **(C)** Similarly, homozygous *kh* mutations reduce daily survival of *Anopheles stephensi* following a blood meal. In this experiment, control [heterozygous (kh^{w+}/kh^{w-})] and homozygous (kh^{w-}/kh^{w-}) mutant mosquitoes were compared. Log-rank tests show a significant difference between survival curves of kh^{w} homozygous mutant females with and their respective controls (kh^{w+}) ($p < 0.0001$ in **B** and **C**).

single amino acid (Y328) and substitution of its neighbor (G329W) at a high functionally-constrained site in the gene that eliminates or greatly reduces KH enzymatic activity when mutated (Gantz et al., 2015). Heterozygous 10.2.18 (kh^{W+}/kh^{W-}) or wild-type *An. stephensi* have black eyes, no fitness or other phenotypes, and were used as controls (Pham et al., 2019). The mosquitoes were maintained at 27 °C with 77% humidity and a 12-h light/dark, 30-min dusk/dawn lighting cycle. Larvae were fed a diet of powdered fish food (Tetra-Min) mixed with yeast. Adults were provided with water and 10% sucrose solution *ad libitum*. Blood meals offered to 4 to 5-day-old females consisted of commercial defibrinated calf blood (Colorado Serum Company, CO, USA) and were provided by using mechanical feeding device (Hemotek, Inc., Blackburn, UK). Aseptic (antibiotic-treated) mosquitoes were reared using a protocol adapted for anophelines (Kumar et al., 2010). *Anopheles stephensi* fourth-instar larvae (50–100 individuals) were transferred to a clean container with 500 ml of an antibiotic solution (10U/mL penicillin/streptomycin in autoclaved water). The solution was changed every day until pupation. Pupae were transferred to a cage with a source of 10% sugar and another mixed antibiotic solution (10U/mL penicillin/streptomycin, 50 μg/ml gentamycin, 200 μg/ml kanamycin). The antibiotic mixture (in a sterile 10% sugar solution) was made fresh for every experiment and kept frozen between changes. The solution was replaced every day until blood feeding (3–5 days after eclosion). The same antibiotic mix used in the sugar solution was added directly to the blood for blood feeding. Experimental feeding supplementation with XA was performed by adding 6 mM XA to the blood. Antioxidant supplementation consisted of mosquitoes being given a 10% sucrose solution with either 25 mg/ml vitamin C or 1 mg/ml uric acid for two days before the blood meal (DeJong et al., 2007).

2.2. Survival analyses

Survival was scored after blood feeding in all of the experiments at 12-h intervals from 0 h to 72 h for *An. stephensi* and daily from 0 h to 25 or 55 days for *Ae. aegypti*. For *Ae. aegypti*, due to the comparatively less deleterious phenotype, multiple blood meals were offered from the first week after adult eclosion. Survival, proportion of successful feeding and oviposition were simultaneously recorded for each blood meal-oviposition cycle. No censoring of outliers was used. Statistical analyses and graph design were performed using Prism 8.0 software (GraphPad Software, San Diego, CA, USA). At least three independent experiments were performed for each treatment (n = 15–20 mosquitoes per experimental group). Data from multiple experiments were combined into a single graph. Kaplan–Meier survival curve analysis (log-rank test) was used to evaluate statistical significance of differences between the experimental and control groups. Survival data are plotted as Kaplan-Meier curves.

2.3. 16S-based analysis of the mosquitoes' microbiota

For *Ae. aegypti* microbiota analysis, mosquitoes were dissected under aseptic conditions consisting of cold anaesthetization, surface sanitization by submersion (2 min each) in 70% ethanol, and rinsing in sterile PBS (1 mM pH 7.4). Midguts were collected using 70% ethanol-cleaned forceps and homogenized in 300 μL of a phenol extraction solution for total RNA extraction by a phenol–chloroform method (Valach, 2016). A total of 1 μg of purified RNA was used in a reverse transcription reaction with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Synthetized cDNA was used as a template in quantitative gene amplification (qPCR)s with universal 16S rRNA bacterial primers. Bacterial 16S rRNA cDNA was amplified by RT-qPCR reactions following a protocol described by Yuen et al. (2002) with minors adaptations: a 40-cycle three-step PCR was performed on a StepOne Plus real-time PCR system (Applied Biosystems) using a 15 μL reaction mix made of 20 mM Tris, pH 8.4, 50 mM KCl, 3 mM MgCl2, 200 μM dNTPs, Reference Dye 1× (ROX, a 100× solution, Sigma-Aldrich), SYBR green 1 0.5 × (stock is a 10,000× solution, Sigma), 200 nM of each primer, and 0.37 units of Jump-start Taq polymerase (Sigma) and 5 μL of cDNA sample (Yuen et al., 2002). The Comparative Ct Method was used (Livak and Schmittgen, 2001) with the ribosomal protein RP49 gene (AAEL003396) as an internal control for the amount of cDNA template (Gentile et al., 2005). Primers used are listed in Table S1. For *An. stephensi*, mosquitoes were dissected under aseptic conditions consisting of cold anaesthetization, surface sanitization by submersion (2–5 min each) in 10% bleach, 70% ethanol, and rinsing in sterile water. Abdomen segments (5–7) were separated using 70% ethanol-cleaned forceps and homogenized in 100 μL sterile distilled water using a motorized pestle. DNA samples were isolated using Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA) according to manufacturer's protocol. Extracted DNA was used as template in qPCRs with universal 16S rRNA bacterial primers (Table S1) designed based on the majority of the mosquito-associated microbiota (γ-*Proteobacteria*). The bacterial 16S rRNA gene from total DNA was amplified by qPCR reactions on an CFX96 Touch™ Real-Time PCR Detection System (Bio--Rad, Hercules, CA, USA). The Comparative Ct Method was used to compare the changes in abundance of target DNA sequences. The ribosomal protein S7 gene was used as internal control for the amount of DNA template. Statistical analyses were performed using a statistical software package (GraphPad Prism 8.0). Two groups (control and mutant) were analyzed using unpaired t tests with Welch's correction for unequal variances when applicable.

2.4. Smurf assay

The loss of midgut barrier function was assayed in *An. stephensi* by feeding them a non-absorbable and non-toxic dye using a protocol adapted from *D. melanogaster* (Rera et al., 2012). Briefly, FD&C blue dye #1 stock solution (320 g/L) was prepared as described (Martins et al., 2018). A feeding solution was prepared using buffered saline (150 mM NaCl/10 mM $NaHCO_3$) (Galun et al., 1985) supplemented with the blue dye (2.5%). Mosquitoes were allowed to feed for 30 min on artificial feeders and fully-engorged females were kept in a cage. Approximately 20 h after feeding, mosquitoes were scored for the 'Smurf' phenotype and separated for bacterial quantification as described above. A female mosquito was counted as a Smurf when blue coloration could be observed outside of the digestive tract. Typical results showed the blue dye retained within the intestine, while in mosquitoes with midgut barrier dysfunction, it was spread throughout the body cavity and visible through the cuticle. The illustrative picture in Fig. S5 shows an optimal representation of a Smurf phenotype, but these can be continuous with lighter shades of blue diffused throughout the body (as noted by Martins et al., 2018). Trained personnel and light microscopy are needed to properly discriminate between experimental groups.

In order to assess whether the dysbiosis observed in homozygous kh^{w} *Ae. aegypti* causes dysfunction of the midgut barrier, a modified version of the Smurf assay protocol was used. The feeding solution was prepared using phosphate-buffered saline (PBS, 137 mM NaCl, 10 mM Na-PO_4 buffer, 2.7 mM KCl/10 mM $NaHCO_3$ - pH 7.4) supplemented with Evans blue dye (1%), Bovine Serum Albumin (1%) and ATP 1 mM. Mosquitoes (4–5 days old) were allowed to feed on artificial feeders and fully engorged females were kept in a cage. Approximately 12 h after feeding, mosquitoes were scored for the Smurf phenotype as described above.

2.5. RNA isolation and quantitative PCR analysis of immune genes

For *Ae. aegypti* immune genes qPCR assays, the RNA of midgut samples was extracted using TRIzol (Thermo Fisher Scientific, Waltham, MA, US) according to the manufacturer's protocol. Complementary DNA was synthesized using the High-Capacity cDNA Reverse transcription kit (Applied Biosystems). The qPCR reactions were performed with the StepOne Plus Real Time PCR System (Applied Biosystems) using the Power SYBR-green PCR master Mix (Applied Biosystems). The

Comparative Ct Method was used to compare the changes in the mRNA accumulation levels and the *Ae. aegypti* RP49 gene was used as an endogenous control (Gentile et al., 2005; Livak and Schmittgen, 2001). Results were analyzed using a statistical software package (GraphPad Prism 8.0).

For *An. stephensi*, whole bodies (5 per sample) of female mosquitoes (24 h after blood feeding) were dissected in PBS, homogenized in TRIzol Reagent (Thermo Fisher Scientific) and total RNA extracted according to the manufacturer's protocol. RNA was treated with the TURBO DNA-free™ Kit (Thermo Fisher Scientific) to remove potential genomic DNA contamination. A total of 1 μg of RNA was used for complementary DNA synthesis using the qScript® cDNA Synthesis Kit (QuantaBio, Beverly, MA, USA). Real-time quantitative PCR reactions were performed with iTaq Universal SYBR Green Supermix (Bio-Rad) and 0.3 μM of each primer (Table S1), and analyzed with a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad), as described above. Fold-changes in mRNA accumulation between blood-fed (relative to sugar-fed) WT and homozygous mutant mosquitoes were derived by the comparative Ct method (Livak and Schmittgen, 2001), using the constitutive ribosomal protein S7 gene as the reference. GraphPad Prism software (8.0) was used to calculate statistical significance using paired t-tests.

3. Results

3.1. Homozygous mutations in the kynurenine hydroxylase-encoding genes decreases lifespan and fertility of adult female mosquitoes

Homozygous mutant kh^{W} *Ae. aegypti* females showed a significant reduction of ~40% in life span ($p < 0.0001$) and lower fecundity, depositing ~30% fewer eggs than wild-type controls ($p < 0.01$) (Fig. 1B; Table 1). Interestingly, the mutant females showed a reduced avidity towards a blood meal ($p < 0.05$), supporting the conclusion that the activity of the KH enzyme has a pleiotropic impact on the physiology of the insect (Table S2). In contrast to the chronic impaired phenotype exhibited by kh^{w} *Aedes* females, an acute effect was observed in the white-eyed *An. stephensi* strain, 10.2.18 (homozygous kh^{w-}/kh^{w-}), with significant high mortality occurring soon after blood feeding (median lethal time, LT_{50}, 36 h–48 h after blood meal; $p < 0.0001$) resulting in a severe reproductive fitness cost (Pham et al., 2019). A *Cx. quinquefasciatus* white-eyed, homozygous kh^{-} line recently developed for functional validation of a Cas9 mutagenesis tool (Feng et al., 2021a, 2021b), exhibited a similar significant reproductive impairment ($p < 0.01$), resulting in fewer hatched larvae ($p < 0.0001$) but did not show acute lethality after blood feeding (Table S3; Fig. S1).

Mass spectrometric analyses of the major KP component metabolites (TRP, 3-HK, KYNA and XA) were performed to further analyze the metabolic profile of the kynurenine pathway in the *An. stephensi* homozygous mutant mosquitoes (Table S4). Surprisingly, the amount of Trp did not differ significantly between mutant and control samples at 24 h after feeding. However, mutant mosquitoes showed only trace amounts of XA ($p < 0.001$). Also, as synthesis of 3-HK was impaired in the mutant mosquitoes, kynurenine formed from tryptophan entering the KP was diverted into kynurenic acid (KYNA), resulting in a significant >3500-fold decrease in the 3-HK/KYNA ratio in mutant mosquitoes compared to controls ($p < 0.001$).

Importantly, the lifespan reduction of *Ae. aegypti* homozygous kh^{W} mutant mosquitoes was reversed significantly when they were fed blood supplemented with XA (Fig. 2A). However, the XA-supplement only restored partially the impact on fecundity (Table 1). Similar experiments with homozygous kh^{W} mutant *An. stephensi* also show a marked reduction of the death rate, achieving levels similar to those found in controls (Fig. 2B). Two different antioxidants, ascorbic acid (vitamin C) and uric acid (DeJong et al., 2007; Graça-Souza et al., 1999) were provided to females in their sugar meals before and after blood feeding to determine if an oxidative imbalance is the cause of the acute mortality of the *An. stephensi* mutant females after feeding. However, this supplementation did not revert the blood meal-induced phenotype supporting the conclusion that the XA-mediated lifespan increase in these mosquitoes is not related to its action as a free-radical scavenger (Fig. S2).

Table 1
Impact of kh^{w} mutations on *Aedes aegypti* egg laying following a blood meal.

	wild-type control	kh^{w}	kh^{w} + XA[a]	kh^{w} + ab[b]
Mean n°. eggs/ female (~150 females)	103.3 ± 4.6 (n = 134)	68.5 ± 4.3[c] (n = 73)	77.6 ± 7.4[d] (n = 92)	77.5 ± 9.5[4] (n = 128)

[a] Supplemented with 6 mM xanthurenic acid (XA).
[b] Supplemented with antibiotics (ab).
[c] $p < 0.01$ (vs. control).
[d] $p < 0.05$ (vs. control).

3.2. kh mutations cause dysbiosis of the midgut microbiota

Microbial abundance in mosquitoes both before and after blood feeding was determined by gene amplification of 16S ribosomal DNA to determine whether the loss of KH activity has a quantitative impact on mosquito midgut microbiota. *Aedes aegypti* wild-type mosquitoes were shown previously to experience a large increase in the midgut bacterial population after blood feeding (Oliveira et al., 2011). Sugar-fed homozygous kh^{w} mutant *Aedes* exhibited an ~10-fold higher basal level of midgut bacteria compared to controls and that increased significantly (25-fold) after a blood meal (Fig. 3A). Heterozygous control (kh^{-}/kh^{+}) *An. stephensi* also showed significant increases in microbiota after a blood meal (Fig. 3B). Remarkably, the homozygous mutant (kh^{w-}/kh^{w-}) *An. stephensi* females, despite the modest and non-significant microbial population proliferation in sugar-fed conditions, showed a significant ~800-fold increase in the size of the microbiota 24 h after feeding on blood (Fig. 3B). In order to assess if the increased microbiota contributed to the loss of lifespan and lethality of the phenotypes in both mosquito species, we treated females with an antibiotic cocktail to deplete bacterial populations from the midgut (Fig. S3). While there was no difference in survival of control mosquitoes following antibiotic treatment, both the shortened lifespan of *Ae. aegypti* and the blood meal-induced lethality phenotype in *An. stephensi* were rescued partially by the microbiota depletion (Fig. 3C and D). Furthermore, XA supplemental feeding reverted the microbiota proliferation in homozygous mutant kh^{W} *Ae. aegypti* blood-fed females (Fig. S4). Together, these results support the conclusion that the loss of fitness seen in mutant mosquitoes is correlated with midgut dysbiosis and implicates a direct role for the kynurenine pathway in the homeostatic regulation of midgut microbiota.

3.3. Homozygous kh^{w} mutation causes midgut barrier dysfunction

A non-invasive method, the so-called 'Smurf assay', was used to monitor midgut barrier status (Martins et al., 2018). This technique assays the permeability of the midgut tissue using a non-absorbable blue dye ('Smurf' phenotype) in orally-fed mosquitoes and can reveal a loss of integrity of the midgut epithelium resulting in leakage of the dye into the hemocoel (Fig. S5). A high proportion of homozygous mutant *An. stephensi* females showed loss of midgut integrity when compared to heterozygous controls (Fig. 4A). Moreover, within the homozygous mutant population, the reduction in survival was observed earlier in Smurf-positive females and was delayed and less severe in mutant females not showing the Smurf phenotype (Fig. 4B). In addition, mutant Smurf-positive females exhibited significantly increased bacterial loads than mutant Smurf-negative females following blood-feeding (Fig. 4C). In contrast, this assay in *Ae. aegypti* did not reveal a measurable difference between mutant and control mosquitoes (Fig. 4D). These results support the conclusion that at least in mutant *An. stephensi*, a

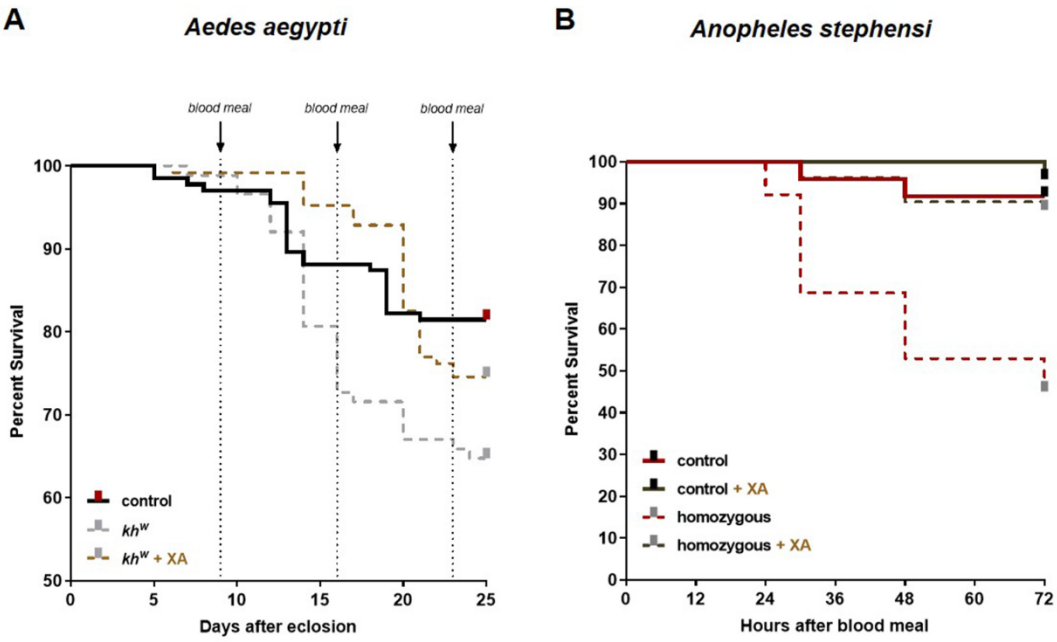

Fig. 2. Xanthurenic acid rescues the homozygous mutant *kh* phenotypes in *Aedes aegypti* and *Anopheles stephensi*. **(A)** Blood meals supplemented with 6 mM xanthurenic acid (XA) restored survival of homozygous mutant kh^{w} *Ae. aegypti* females. Supplemented (+XA) and control (no XA) blood meals offered as indicated. The probability of survival (y-axis) was scored for 25 days (x-axis) after feeding and the data plotted as Kaplan-Meier survival curves. **(B)** Similarly, blood meals supplemented with 6 mM XA restored survival of homozygous mutant *An. stephensi* females. As in Fig. 1, control [heterozygous (kh^{w+}/kh^{w-})] and homozygous (kh^{w-}/kh^{w-}) -mutant mosquitoes were compared and the probability of survival over 72 h after feeding plotted. Data from three or more independent experiments (n = 15–20 mosquitoes per experimental group) were combined into a single graph for both A and B. Log-rank tests show a significant difference between survival curves of kh^{w} mutant females with and without XA supplementation ($p < 0.05$ in **A**; $p < 0.001$ in **B**).

combination of an expansion of the midgut microbial population and midgut barrier dysfunction may drive the acute mortality that occurs after blood feeding in individuals deficient for KH function.

Given the magnitude of the change in the midgut microbial population compared to control mosquitoes after a blood meal intake, and the existence of a pathological condition elicited by this microbial overload, we hypothesized that a transcriptional immune response could be activated in mutant females. We evaluated the mRNA levels of a set of antimicrobial peptide genes in both *Ae. aegypti* and *An. stephensi*. Surprisingly, with the exception of the cecropin-encoding gene ortholog in *An. stephensi*, there was no significant effect of the mutations on the mRNA levels of these immune genes (Fig. 5A and B). We interpret this to indicate a direct, non-canonical involvement of KP metabolites in microbiota control and maintenance of physiological homeostasis in blood-feeding mosquitoes.

4. Discussion

The development of technologies for insect genetic engineering have long made use of pigmentation genes that when altered lead to visible, easy-to-screen phenotypes. In the recent years, the advancement of self-propagating gene drive systems made it feasible to implement the use of genetic alterations to effectively reduce or eliminate insect-borne diseases (Caragata et al., 2020; Wang et al., 2021). The results described herein provide useful information for mutagenesis of a common target of modification, the *kh* gene family of orthologs, in three mosquito species. Given the high functional constraints of the targeted genomic site within the *kh* gene, mutations in the genes of all three mosquito species result in loss-of-function and distinctive white-eye phenotypes observable during larval, pupal and adult stages (Feng et al., 2021a; Gantz et al., 2015; Han et al., 2003). Notably, in addition to these visible features, we present here unanticipated roles of KP metabolites in maintaining homeostasis in these organisms following blood feeding, especially in implicating the KP as a regulator of the mosquito–microbiota interaction.

Eye-color mutations in *D. melanogaster* are often used as tools for study of neurodegenerative diseases (Green et al., 2012). For example, in a vinegar fly model of Huntington's disease, small-molecule or genetic inhibition of *kh*-encoded activity increases the levels of neuroprotective KYNA, decreases the levels of neurotoxic 3-HK, and reduces neurodegeneration (Campesan et al., 2011). Down-regulation of Trp-Kyn metabolism is associated with a prolongation of life span in flies carrying eye-color mutations (Oxenkrug, 2010). Transamination of the chemically-reactive 3-HK to the chemically-stable XA is a major branch pathway of Trp metabolism in mosquitoes (Han et al., 2007). Conversely, we found in mutant *An. stephensi* and *Ae. aegypti* (Lima et al., 2012) that a decrease in flux of the central KP, with the accumulation of KYNA, negatively impacts their survival. While food intake, fecundity and fertility are impaired significantly in *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*, no similar phenotypes have been associated with similar mutations in non-blood feeding insects (Summers et al., 1982). Additionally, the *kh* mutant mosquitoes examined in this study show no obvious differences in premature stages or male survival. Interestingly, it was shown recently that blood feeding is lethal for a *cinnabar* (*kh*) knockdown model in the kissing bug *Rhodnius prolixus*, resulting in death of all young animals before molting (Berni et al., 2020). In mosquitoes homozygous for *kh* mutations, XA formation is severely impaired, resulting in increased midgut cell death in *Ae. aegypti* that was linked tentatively to heme, metal-chelating and antioxidant activities of this metabolite (Christen et al. 1990; Murakami et al. 2001; Lima et al., 2012). However, the inability of antioxidant supplementation in attenuating the mutant *kh* fitness loss as shown here makes it unlikely that the XA protective effect in mosquito physiology could be due to its redox properties.

Regulatory and functional aspects of the KP are recognized more widely in mammals where it controls Trp detoxification and availability for synthesis of serotonin and other neuroactive metabolites (Badawy, 2017). The KP is regulated by, and in turn regulates, different physiological systems that are disrupted commonly in neurological disorders,

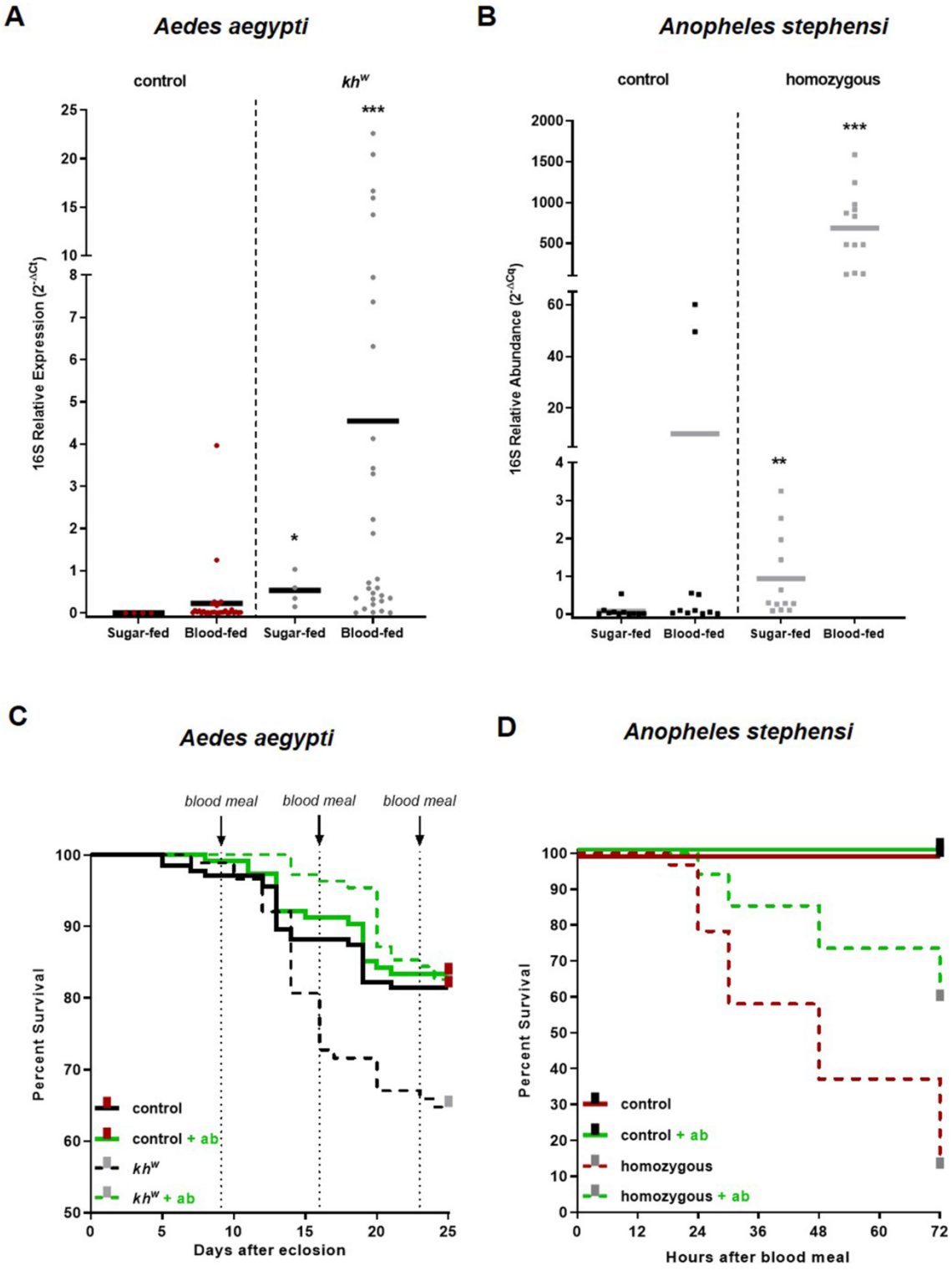

Fig. 3. Proliferation of midgut microbiota is associated with decreased survival of homozygous mutant *kh* mosquitoes. (A, B) The relative abundance (y-axis) of bacterial ribosomal 16S DNA following gene amplification of samples from **(A)** *Aedes aegypti* and **(B)** *Anopheles stephensi* shows increased bacterial proliferation in homozygous mutant *kh* mosquitoes. The columns show the combined results of at least three independent experiments with samples from individual sugar-fed and blood-fed, control and homozygous mutant mosquitoes. Statistical analyses were performed using t tests between control and homozygous mutant mosquito corresponding samples (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). As in previous experiments, wild-type *Ae. aegypti* and heterozygous mutant *kh An. stephensi* served as the respective controls. **(C)** *Aedes aegypti* homozygous kh^w mutant mosquitoes treated with antibiotics for three days before each of the three weekly blood meals had greater probabilities of survival (y-axis) through 25 days (x-axis) than untreated mutant mosquitoes in at least two independent experiments (n = 15–20 mosquitoes per experimental group). Untreated control (wild-type) and mutant mosquitoes served as treatment controls. Survival data are plotted as Kaplan-Meier curves. **(D)** Similarly-treated *An. stephensi* homozygous kh^{w-} mutant mosquitoes also showed increases in survival probability. Control [heterozygous (kh^{w+}/kh^{w-})] mosquitoes served as treatment controls. Data from the multiple experiments were combined into a single graph in all panels. Log-rank tests show a significant difference between survival curves of kh^w mutant females with and without antibiotic treatment ($p < 0.01$ in **C**; $p < 0.0001$ in **D**).

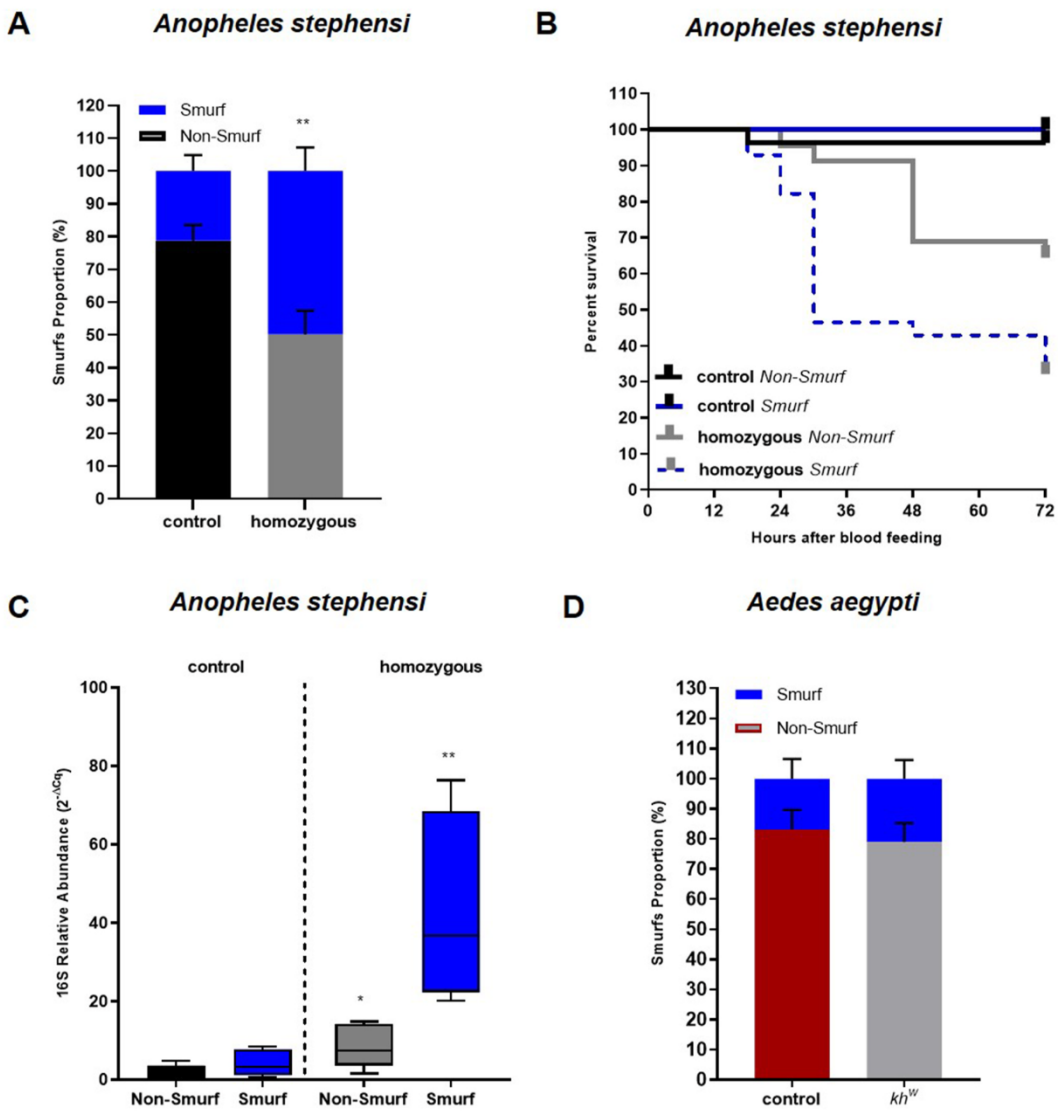

Fig. 4. Homozygous *kh*⁻ mutations induce midgut integrity dysfunction in *An. stephensi* but not in *Ae. aegypti*. **(A)** Homozygous kh^{-} mutations in *An. stephensi* induce increases in midgut permeability. The proportion of control [heterozygous (kh^{w+}/kh^{w-})] and homozygous (kh^{w-}/kh^{w-}) mutant female mosquitoes showing loss of midgut integrity ('Smurf' assay, SI Fig. 5) assayed using blue dye n°1 (**$p < 0.01$). (B) Survival after blood feeding of control (kh^{w+}/kh^{w-}) and homozygous (kh^{w-}/kh^{w-}) mutant female mosquitoes grouped as non-Smurfs or Smurfs, two days after blue dye-feeding. (C) Abundance of bacterial ribosomal 16S DNA qPCR in control [heterozygous (kh^{w+}/kh^{w-})] and homozygous (kh^{w-}/kh^{w-}), grouped as non-Smurfs or Smurfs depending on their phenotype. At least three independent experiments with 15–20 individuals per experiment are shown. Statistical analyses were performed using Student's *t*-test between corresponding control (heterozygous) and homozygous samples (*$p < 0.05$, **$p < 0.01$). (D) Score of Smurf phenotype proportion (y-axis) between control (wild-type) and homozygous kh^{w} mutant *Ae. aegypti female* mosquitoes. Statistical analyses were performed using Student's *t*-test and the proportion values between experimental groups are non-significant.

including hormonal and metabolic systems, thus impacting cognition, pain, metabolism, and aging (Savitz, 2020). Levels of KP enzymes are regulated by steroids, growth factors and, most notoriously, signaling molecules of the immune system, and several studies have shown that the catabolism of dietary tryptophan regulate inflammation and disease development (Salter and Pogson 1985; Belladonna et al., 2006; Grifka-Walk et al. 2021). The presumed existence of a kynurenine receptor that is a component of a xenobiotic response element, suggests that kynurenines may be natural endogenous ligands of a protective signaling pathway, reducing illness triggered or caused by chemicals, and even prolonging survival (Opitz et al., 2011; Stone et al. 2013). More relevant here, KP intermediates were suggested as promoters of tolerance to bacterial-induced inflammation through a signaling cascade involving the aryl hydrocarbon receptor (AHR), the putative kynurenine receptor (Bessede et al., 2014). Furthermore, endogenous and bacterial Trp metabolites, acting through a kynurenine-specific signaling pathway, affect gut microbial composition and the host immune system–midgut microbiota interactions through fine-tuning of the balance between midgut immune tolerance and gut microbiota maintenance (Gao et al., 2018; Laurans et al., 2018).

While being recognized widely as having a role in the regulation of homeostasis, the indigenous microbiota also may be an important causative factor of midgut damage under pathologic conditions. In mosquitoes, a considerable microbiota proliferation and reduction in bacterial diversity is observed after a blood meal (Pumpuni et al., 1996; Terenius et al., 2012). The fine balance of the midgut microbial community is of paramount importance to the physiology of hematophagic insect vectors, affecting development, digestion, reproduction, and immunity. Here we found that a common key feature of *kh* disruptions is a loss of microbiota proliferation control after feeding, leading to a quantitative increase in midgut bacteria abundance. Specifically, the midgut dysbiosis in *An. stephensi* combined with increased midgut

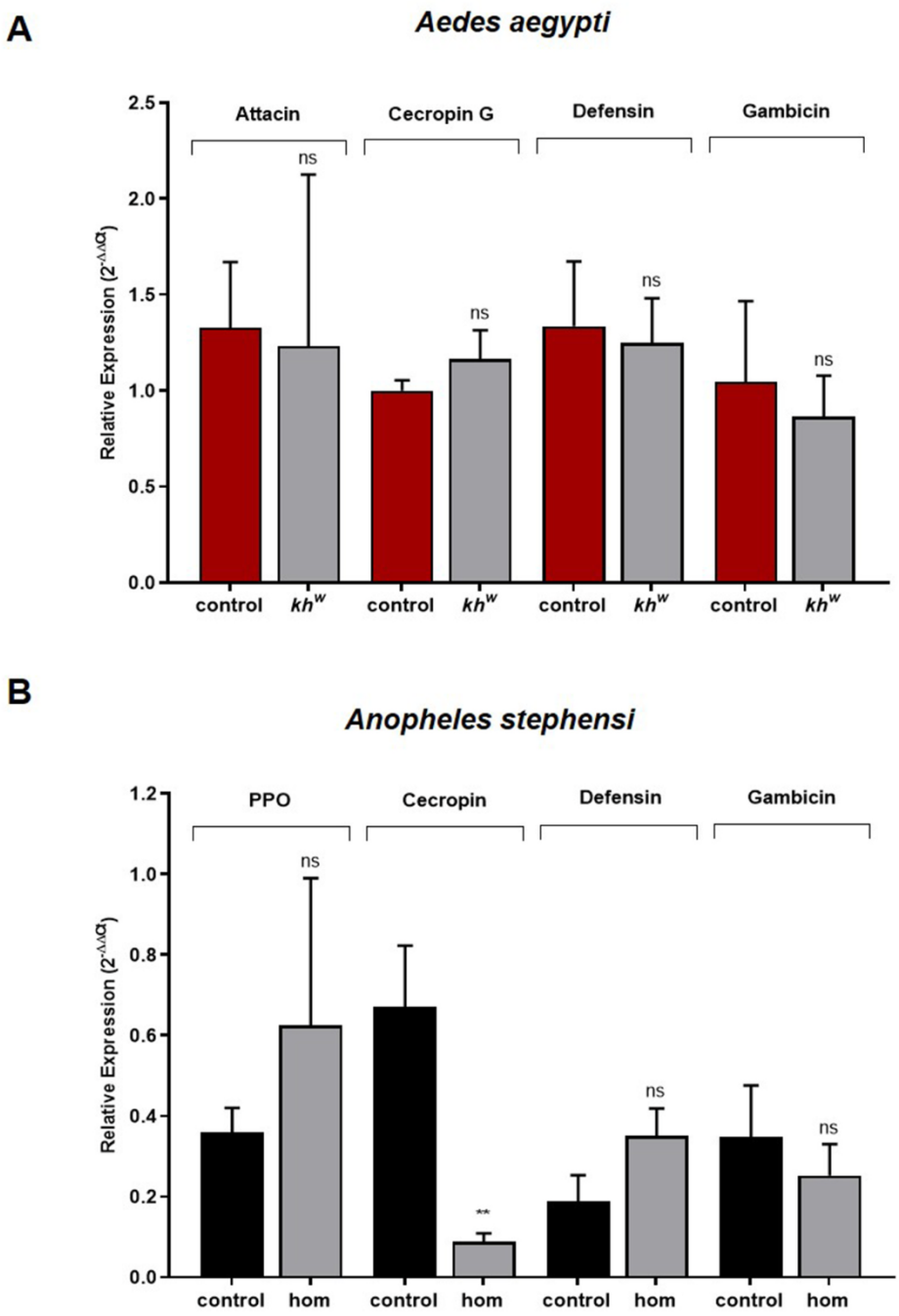

Fig. 5. Homozygous kh^w mutations do not result in a general altered immune response profile. Relative immune gene RNA expression levels in (A) *Ae. aegypti* and (B) *An. stephensi* control (wild-type or heterozygous, respectively) and homozygous kh^w mutant female mosquitoes, 24 h after blood feeding. Statistical analyses performed using Student's *t*-test between corresponding control and homozygous mutant samples (ns = non-significant, $^{**}p < 0.01$).

epithelial permeability is a likely cause of the severe lethal phenotype after blood feeding characterized by an early onset mortality similarly to that reported in age-related midgut barrier dysfunction in *Drosophila* (Clark et al., 2015; Rera et al., 2012). Midgut integrity in 3–7 days-old *Ae. aegypti* appears equal between *kh* mutant and control individuals. This may explain the relatively milder phenotype presented in this species, defined by the age-onset shortening of life span associated with the *kh* mutation. Namely, there appears to be a link between loss-of-function of *kh*-encoded products and age-related mortality, given that the deleterious phenotype is observed to a greater extent in 10 day and older mosquitoes and increasingly after multiple blood meals. Therefore, a cumulative damage might be in place. Further research is required to explore this and other potential explanations. Whatever the cause of the differences between the phenotypes presented by these two species, a common aspect is the occurrence of dysbiosis as revealed by rescue via antibiotic treatment. When blood-fed mutant and control mosquitos were compared, given the magnitude of increase in the microbial population in both cases (25× in *Aedes* and 70× in *Anopheles*) and the clear loss of fitness, an activation of an immune response would be expected, especially for the *An. stephensi*, where the barrier function of the midgut wall has been compromised. However, a noticeable feature in both species was the absence of relevant alteration of expression of selected immune genes, with the cecropin gene in *An. stephensi* being the notable exception, suggesting a general lack of

immune signaling activation. A more comprehensive survey of the immune system genes may provide more information on this observation.

Although midgut composition analyses of microbial species in mosquitoes most frequently display a low-diversity community (Coon et al., 2014; Sterkel et al., 2017), it is not known whether specific components of the midgut microbiota play a role in the observed physiology of *kh* mutant mosquitoes. It is important to emphasize that this study used bacterial quantification with 16S qPCR due to its technical advantages, such as utilization of small template amounts, high sensitivity, and large throughput processing. However, differential selection of bacterial species that could promote pathogenicity among the indigenous microbiota is a likely mechanism for dysbiosis, and the nature of these putative pathobionts could account for the differences in fitness cost severity between the distinct mosquito species studied here. Therefore, additional studies are needed to indicate how shifts in gut community composition (complexity) and/or abundance affect the fitness of females across different mosquito species. Furthermore, given that initial colonization events are determinants of adult microbiota composition (Coon et al., 2014), it is possible that environmental differences in mosquito rearing (e.g. field vs. laboratory-reared, or even different food sources for larval nutrition) could account for variations on the impact of such microbiome-relevant mutations, even within the same species. For example, Yamamoto et al. (2018) were able to generate a viable *kh* homozygous mutant *An. stephensi* line, using a TALEN mutagenesis system, with no reported survival or reproductive fitness cost. It is possible that differences in the midgut microbiota of those individuals or other aspects of the feeding regimen may have supplied sufficient dietary supplementation to rescue a potentially-lethal phenotype.

Furthermore, a genetic phenotype rescue system was developed in *An. stephensi* with the use of a recoded *kh* sequence that supports normal survival and reproductive capacity in females, while eliminating from the population individuals carrying nonfunctional mutated kh^{w} copies or failing to inherit the recoded construct (Adolfi et al., 2020). We also have recently generated a Cas9/gRNA-based gene drive system in *An. gambiae* that targets the *cardinal* gene ortholog, disrupting the conversion of 3-HK to xanthommatin and producing a red-eye phenotype (Carballar-Lejarazú et al., 2020). This next-generation gene drive strain has no major fitness load and presents a low-frequency formation of potential resistant alleles. Nonetheless, it is important to point out that empirical studies done in laboratory mosquito populations should carefully consider the physiological effect of a given genetic alteration in different subpopulations, and ideally be tested in multiple intercrossing (e.g., genetic introduction) and natural settings.

In summary, we demonstrate that *kh*-knockout mutations are not only associated with a deficiency in 3-HK and resulting white-eye phenotypes, but also to an altered microbiota phenotype, impacting multiple aspects of mosquito physiology, across different species. Our data support a model whereby KH enzymatic impairment and consequent XA deficiency led to expedited mortality after blood feeding, coordinated with a great bacterial growth and systemic homeostasis dysfunction. Therefore, aside from its role as a genetic marker, *kh* mutations are useful experimental systems for analyzing the effects of Trp metabolites on important aspects of vector biology. It is also hoped that this work will provide an incentive for those researchers developing population modification strategies to comprehensively approach the effect of a given proposed alteration and better predict the impact of genetic interventions. The description of the phenotypes associated with the genetic ablation of a central KP branch in mosquitoes presented here provides a foundation for future studies seeking to identify specific gene families, molecules and pathways that support key regulatory and functional aspects of the KP in blood-feeding vectors.

Funding

Funding was provided by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and the University of California Irvine Malaria Initiative. AAJ is a Donald Bren Professor at the University of California, Irvine.

Data availability

Data will be made available on request.

Acknowledgments

We are grateful to the members of the UCI insectary staff who provided excellent support in mosquito husbandry, and to J Loredo, C Cosme, J Marques and SR Cássia for technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2022.103720.

References

Adolfi, A., Gantz, V.M., Jasinskiene, N., Lee, H., Hwang, K., Terradas, G., Bulger, E.A., Ramaiah, A., Bennett, J.B., Emerson, J.J., Marshall, J.M., Bier, E., James, A.A., 2020. Efficient population modification gene-drive rescue system in the malaria mosquito *Anopheles stephensi*. Nat. Commun. 11, 5553. https://doi.org/10.1038/s41467-020-19426-0.

Aryan, A., Anderson, M.A.E., Myles, K.M., Adelman, Z.N., 2013. TALEN-based gene disruption in the dengue vector *Aedes aegypti*. PLoS One 8. https://doi.org/10.1371/journal.pone.0060082.

Badawy, A.A., 2017. Kynurenine pathway of tryptophan metabolism: regulatory and functional aspects. Int. J. Tryptophan Res. 10 https://doi.org/10.1177/1178646917691938, 117864691769193.

Basu, S., Aryan, A., Overcash, J.M., Samuel, G.H., Anderson, M.A.E., Dahlem, T.J., Myles, K.M., Adelman, Z.N., 2015. Silencing of end-joining repair for efficient site-specific gene insertion after TALEN/CRISPR mutagenesis in *Aedes aegypti*. Proc. Natl. Acad. Sci. U.S.A. 112, 4038–4043. https://doi.org/10.1073/pnas.1502370112.

Belladonna, M.L., Grohmann, U., Guidetti, P., Volpi, C., Bianchi, R., Fioretti, M.C., Schwarcz, R., Fallarino, F., Puccetti, P., 2006. Kynurenine pathway enzymes in dendritic cells initiate tolerogenesis in the absence of functional Ido. J. Immunol. 177, 130–137. https://doi.org/10.4049/jimmunol.177.1.130.

Berni, M., Bressan, D., Simão, Y., Julio, A., Oliveira, P.L., Pane, A., Araujo, H., 2020. Pigmentation loci as markers for genome editing in the Chagas disease vector. Rhodnius prolixus. https://doi.org/10.1101/2020.04.29.067934 bioRxiv 2020.04.29.067934.

Bessede, A., Gargaro, M., Pallotta, M.T., Matino, D., Servillo, G., Brunacci, C., Bicciato, S., Mazza, E.M.C., Macchiarulo, A., Vacca, C., Iannitti, R., Tissi, L., Volpi, C., Belladonna, M.L., Orabona, C., Bianchi, R., Lanz, T.V., Platten, M., Della Fazia, M.A., Piobbico, D., Zelante, T., Funakoshi, H., Nakamura, T., Gilot, D., Denison, M.S., Guillemin, G.J., DuHadaway, J.B., Prendergast, G.C., Metz, R., Geffard, M., Boon, L., Pirro, M., Iorio, A., Veyret, B., Romani, L., Grohmann, U., Fallarino, F., Puccetti, P., 2014. Aryl hydrocarbon receptor control of a disease tolerance defence pathway. Nature 511, 184–190. https://doi.org/10.1038/nature13323.

Bhalla, S.C., 1968. White eye, a new sex-linked mutant of *Aedes aegypti*. Mosq. News 28, 380–385.

Bonizzoni, M., Dunn, W.A., Campbell, C.L., Olson, K.E., Dimon, M.T., Marinotti, O., James, A.A., 2011. RNA-seq analyses of blood-induced changes in gene expression in the mosquito vector species, *Aedes aegypti*. BMC Genom. 12, 82. https://doi.org/10.1186/1471-2164-12-82.

Bottino-Rojas, V., Talyuli, O.A.C., Jupatanakul, N., Sim, S., Dimopoulos, G., Venancio, T. M., Bahia, A.C., Sorgine, M.H., Oliveira, P.L., Paiva-Silva, G.O., 2015. Heme signaling impacts global gene expression, immunity and dengue virus infectivity in *Aedes aegypti*. PLoS One 10, e0135985. https://doi.org/10.1371/journal.pone.0135985.

Campesan, S., Green, E.W., Breda, C., Sathyasaikumar, K.V., Muchowski, P.J., Schwarcz, R., Kyriacou, C.P., Giorgini, F., 2011. The kynurenine pathway modulates neurodegeneration in a Drosophila model of Huntington's disease. Curr. Biol. 21, 961–966. https://doi.org/10.1016/j.cub.2011.04.028.

Caragata, E.P., Dong, S., Dong, Y., Simões, M.L., Tikhe, C.V., Dimopoulos, G., 2020. Prospects and pitfalls: next-generation tools to control mosquito-transmitted disease. Annu. Rev. Microbiol. 74, 455–475. https://doi.org/10.1146/annurev-micro-011320-025557.

Carballar-Lejarazú, R., Ogaugwu, C., Tushar, T., Kelsey, A., Pham, T.B., Murphy, J., Schmidt, H., Lee, Y., Lanzaro, G.C., James, A.A., 2020. Next-generation gene drive for population modification of the malaria vector mosquito, *Anopheles gambiae*. Proc. Natl. Acad. Sci. U.S.A. 117, 22805–22814. https://doi.org/10.1073/pnas.2010214117.

Christen, S., Peterhans, E., Stocker, R., 1990. Antioxidant activities of some tryptophan metabolites: possible implication for inflammatory diseases (influenza/interferon-γ/oxidative stress/indoleamine 2,3-dioxygenase/3-hydroxykynurenine). Proc. Natl. Acad. Sci. U.S.A. 87, 2506–2510.

Clark, R.I., Salazar, A., Yamada, R., Fitz-Gibbon, S., Morselli, M., Alcaraz, J., Rana, A., Rera, M., Pellegrini, M., Ja, W.W., Walker, D.W., 2015. Distinct shifts in microbiota composition during Drosophila aging impair intestinal function and drive mortality. Cell Rep. 12, 1656–1667. https://doi.org/10.1016/j.celrep.2015.08.004.

Coates, C.J., Jasinskiene, N., Miyashiro, L., James, A.A., 1998. Mariner transposition and transformation of the yellow fever mosquito, *Aedes aegypti*. Proc. Natl. Acad. Sci. U.S.A. 95, 3748–3751. https://doi.org/10.1073/pnas.95.7.3748.

Coon, K.L., Vogel, K.J., Brown, M.R., Strand, M.R., 2014. Mosquitoes rely on their gut microbiota for development. Mol. Ecol. 23, 2727–2739. https://doi.org/10.1111/mec.12771.

Cornel, A.J., Benedict, M.Q., Rafferty, C.S., Howells, A.J., Collins, F.H., 1997. Transient expression of the *Drosophila melanogaster* cinnabar gene rescues eye color in the white eye (WE) strain of *Aedes aegypti*. Insect Biochem. Mol. Biol. 27, 993–997. https://doi.org/10.1016/S0965-1748(97)00084-2.

DeJong, R.J., Miller, L.M., Molina-Cruz, A., Gupta, L., Kumar, S., Barillas-Mury, C., 2007. Reactive oxygen species detoxification by catalase is a major determinant of fecundity in the mosquito *Anopheles gambiae*. Proc. Natl. Acad. Sci. U.S.A. 104, 2121–2126. https://doi.org/10.1073/pnas.0608407104.

Dermauw, W., Van Leeuwen, T., 2014. The ABC gene family in arthropods: comparative genomics and role in insecticide transport and resistance. Insect Biochem. Mol. Biol. 45, 89–110. https://doi.org/10.1016/j.ibmb.2013.11.001.

Feng, X., Kambic, L., Nishimoto, J.H.K., Reed, F.A., Denton, J.A., Sutton, J.T., Gantz, V. M., 2021a. Evaluation of Gene Knockouts by CRISPR as potential targets for the genetic engineering of the mosquito *Culex quinquefasciatus*. Cris. J. 4, 595–608. https://doi.org/10.1089/crispr.2021.0028.

Feng, X., López Del Amo, V., Mameli, E., Lee, M., Bishop, A.L., Perrimon, N., Gantz, V.M., 2021b. Optimized CRISPR tools and site-directed transgenesis towards gene drive development in *Culex quinquefasciatus* mosquitoes. Nat. Commun. 12 https://doi.org/10.1038/s41467-021-23239-0.

Figon, F., Casas, J., 2019. Ommochromes in invertebrates: biochemistry and cell biology. Biol. Rev. 94, 156–183. https://doi.org/10.1111/brv.12441.

Galun, R., Koontz, L.C., Gwadz, R.W., 1985. Engorgement response of anopheline mosquitoes to blood fractions and artificial solutions. Physiol. Entomol. 10, 145–149. https://doi.org/10.1111/j.1365-3032.1985.tb00029.x.

Gantz, V.M., Bier, E., 2015. The mutagenic chain reaction: a method for converting heterozygous to homozygous mutations. Science 348, 442–444. https://doi.org/10.1126/science.aaa5945.

Gantz, V.M., Jasinskiene, N., Tatarenkova, O., Fazekas, A., Macias, V.M., Bier, E., James, A.A., 2015. Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. Proc. Natl. Acad. Sci. U.S.A. 112, E6736–E6743. https://doi.org/10.1073/pnas.1521077112.

Gao, J., Xu, K., Liu, H., Liu, G., Bai, M., Peng, C., Li, T., Yin, Y., 2018. Impact of the gut microbiota on intestinal immunity mediated by tryptophan metabolism. Front. Cell. Infect. Microbiol. 8, 1–22. https://doi.org/10.3389/fcimb.2018.00013.

Garcia, E.S., Macarini, J.D., Garcia, M.L., Ubatuba, F.B., 1975. Feeding of *Rhodnius prolixus* in the laboratory. An. Acad. Bras. Cienc. 47, 537–545.

Gentile, C., Lima, J.B.P., Peixoto, A.A., 2005. Isolation of a fragment homologous to the rp49 constitutive gene of Drosophila in the Neotropical malaria vector *Anopheles aquasalis* (Diptera: Culicidae). Mem. Inst. Oswaldo Cruz 100, 545–547. https://doi.org/10.1590/S0074-02762005000600008.

Graça-Souza, A.V., Silva-Neto, M.A.C., Oliveira, P.L., 1999. Urate synthesis in the blood-sucking insect *Rhodnius prolixus*. J. Biol. Chem. 274, 9673–9676. https://doi.org/10.1074/jbc.274.14.9673.

Green, E.W., Campesan, S., Breda, C., Sathyasaikumar, K.V., Muchowski, P.J., Schwarcz, R., Kyriacou, C.P., Giorgini, F., 2012. Drosophila eye color mutants as therapeutic tools for Huntington disease. Fly (Austin) 6, 117–120. https://doi.org/10.4161/fly.19999.

Grifka-Walk, H.M., Jenkins, B.R., Kominsky, D.J., 2021. Amino acid trp: the far out impacts of host and commensal tryptophan metabolism. Front. Immunol. 12, 1–14. https://doi.org/10.3389/fimmu.2021.653208.

Han, Q., Beerntsen, B.T., Li, J., 2007. The tryptophan oxidation pathway in mosquitoes with emphasis on xanthurenic acid biosynthesis. J. Insect Physiol. 53, 254–263. https://doi.org/10.1016/j.jinsphys.2006.09.004.

Han, Q., Calvo, E., Marinotti, O., Fang, J., Rizzi, M., James, A.A., Li, J., 2003. Analysis of the wild-type and mutant genes encoding the enzyme kynurenine monooxygenase of the yellow fever mosquito, *Aedes aegypti*. Insect Mol. Biol. 12, 483–490. https://doi.org/10.1046/j.1365-2583.2003.00433.x.

Jasinskiene, N., Coates, C.J., Benedict, M.Q., Cornel, A.J., Rafferty, C.S., James, A.A., Collins, F.H., 1998. Stable transformation of the yellow fever mosquito, *Aedes aegypti*, with the Hermes element from the housefly. Proc. Natl. Acad. Sci. U.S.A. 95, 3743–3747. https://doi.org/10.1073/pnas.95.7.3743.

Jiang, X., Peery, A., Hall, A.B., Sharma, A., Chen, X.-G., Waterhouse, R.M., Komissarov, A., Riehle, M.M., Shouche, Y., Sharakhova, M.V., Lawson, D., Pakpour, N., Arensburger, P., Davidson, V.L.M., Eiglmeier, K., Emrich, S., George, P., Kennedy, R.C., Mane, S.P., Maslen, G., Oringanje, C., Qi, Y., Settlage, R., Tojo, M., Tubio, J.M.C., Unger, M.F., Wang, B., Vernick, K.D., Ribeiro, J.M.C., James, A.A., Michel, K., Riehle, M.A., Luckhart, S., Sharakhov, I.V., Tu, Z., 2014. Genome analysis of a major urban malaria vector mosquito, *Anopheles stephensi*. Genome Biol. 15, 459. https://doi.org/10.1186/s13059-014-0459-2.

Kokoza, V., Ahmed, A., Wimmer, E.A., Raikhel, A.S., 2001. Efficient transformation of the yellow fever mosquito *Aedes aegypti* using the piggyBac transposable element vector pBac[3xP3-EGFP afm]. Insect Biochem. Mol. Biol. 31, 1137–1143. https://doi.org/10.1016/S0965-1748(01)00120-5.

Kumar, S., Molina-Cruz, A., Gupta, L., Rodrigues, J., Barillas-Mury, C., 2010. A peroxidase/dual oxidase system modulates epithelial immunity in *Anopheles gambiae*. Science (80-.) 327, 1644–1649. https://doi.org/10.1126/science.1184008.

Laurans, L., Venteclef, N., Haddad, Y., Chajadine, M., Alzaid, F., Metghalchi, S., Sovran, B., Denis, R.G.P., Dairou, J., Cardellini, M., Moreno-Navarrete, J.M., Straub, M., Jegou, S., McQuitty, C., Viel, T., Esposito, B., Tavitian, B., Callebert, J., Luquet, S.H., Federici, M., Fernandez-Real, J.M., Burcelin, R., Launay, J.M., Tedgui, A., Mallat, Z., Sokol, H., Taleb, S., 2018. Genetic deficiency of indoleamine 2,3-dioxygenase promotes gut microbiota-mediated metabolic health. Nat. Med. 24, 1113–1120. https://doi.org/10.1038/s41591-018-0060-4.

Li, M., Bui, M., Yang, T., Bowman, C.S., White, B.J., Akbari, O.S., 2017. Germline Cas9 expression yields highly efficient genome engineering in a major worldwide disease vector, *Aedes aegypti*. Proc. Natl. Acad. Sci. U.S.A. 114 (49), E10540–E10549. https://doi.org/10.1073/pnas.1711538114, 201711538.

Lima, V.L.A., Dias, F., Nunes, R.D., Pereira, L.O., Santos, T.S.R., Chiarini, L.B., Ramos, T. D., Silva-Mendes, B.J., Perales, J., Valente, R.H., Oliveira, P.L., 2012. The antioxidant role of xanthurenic acid in the *Aedes aegypti* midgut during digestion of a blood meal. PLoS One 7, 1–8. https://doi.org/10.1371/journal.pone.0038349.

Liu, T., Yang, W.Q., Xie, Y.G., Liu, P.W., Xie, L.H., Lin, F., Li, C.Y., Gu, J.B., Wu, K., Yan, G.Y., Chen, X.G., 2019. Construction of an efficient genomic editing system with CRISPR/Cas9 in the vector mosquito Aedes albopictus. Insect Sci. 26, 1045–1054. https://doi.org/10.1111/1744-7917.12645.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ÄÄCT method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.

Marinotti, O., Calvo, E., Nguyen, Q.K., Dissanayake, S., Ribeiro, J.M.C., James, A.A., 2006. Genome-wide analysis of gene expression in adult *Anopheles gambiae*. Insect Mol. Biol. 15, 1–12. https://doi.org/10.1111/j.1365-2583.2006.00610.x.

Martins, R., McCracken, A., Simons, M., Henriques, C., Rera, M., 2018. How to catch a Smurf? – ageing and Beyond…In vivo assessment of intestinal permeability in multiple model organisms. Bio-Protocol 8. https://doi.org/10.21769/bioprotoc.2722.

Murakami, K., Ito, M., Yoshino, M., 2001. Xanthurenic acid inhibits metal ion-induced lipid peroxidation and protects NADP-isocitrate dehydrogenase from oxidative inactivation. J. Nutr. Sci. Vitaminol. (Tokyo). 47, 306–310. https://doi.org/10.3177/jnsv.47.306.

Oliveira, J.H.M., Gonçalves, R.L.S., Lara, F.A., Dias, F.A., Gandara, A.C.P., Menna-Barreto, R.F.S., Edwards, M.C., Laurindo, F.R.M., Silva-Neto, M.A.C., Sorgine, M.H. F., Oliveira, P.L., 2011. Blood meal-derived heme decreases ROS levels in the midgut of *Aedes aegypti* and allows proliferation of intestinal microbiota. PLoS Pathog. 7 https://doi.org/10.1371/journal.ppat.1001320.

Opitz, C.A., Litzenburger, U.M., Sahm, F., Ott, M., Tritschler, I., Trump, S., Schumacher, T., Jestaedt, L., Schrenk, D., Weller, M., Jugold, M., Guillemin, G.J., Miller, C.L., Lutz, C., Radlwimmer, B., Lehmann, I., von Deimling, A., Wick, W., Platten, M., 2011. An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. Nature 478, 197–203. https://doi.org/10.1038/nature10491.

Oxenkrug, G.F., 2010. The extended life span of *Drosophila melanogaster* eye-color (white and vermilion) mutants with impaired formation of kynurenine. J. Neural. Transm. 117, 23–26. https://doi.org/10.1007/s00702-009-0341-7.

Oxenkrug, G.F., Navrotskaya, V., Voroboyva, L., Summergrad, P., 2011. Extension of life span of *Drosophila melanogaster* by the inhibitors of tryptophan-kynurenine metabolism. Fly (Austin) 5, 307–309. https://doi.org/10.4161/fly.5.4.18414.

Pham, T.B., Phong, C.H., Bennett, J.B., Hwang, K., Jasinskiene, N., Parker, K., Stillinger, D., Marshall, J.M., Carballar-Lejarazú, R., James, A.A., 2019. Experimental population modification of the malaria vector mosquito, *Anopheles stephensi*. PLoS Genet. 15, e1008440 https://doi.org/10.1371/journal.pgen.1008440.

Pumpuni, C.B., Demaio, J., Kent, M., Davis, J.R., Beier, J.C., 1996. Bacterial population dynamics in three anopheline species: the impact on Plasmodium sporogonic development. Am. J. Trop. Med. Hyg. 54, 214–218. https://doi.org/10.4269/ajtmh.1996.54.214.

Rera, M., Clark, R.I., Walker, D.W., 2012. Intestinal barrier dysfunction links metabolic and inflammatory markers of aging to death in Drosophila. Proc. Natl. Acad. Sci. U.S. A. 109, 21528–21533. https://doi.org/10.1073/pnas.1215849110.

Rivera-Pérez, C., Clifton, M.E., Noriega, F.G., 2017. How micronutrients influence the physiology of mosquitoes. Curr. Opin. Insect Sci. 23, 112–117. https://doi.org/10.1016/j.cois.2017.07.002.

Salter, M., Pogson, C.I., 1985. The role of tryptophan 2,3-dioxygenase in the hormonal control of tryptophan metabolism in isolated rat liver cells. Effects of glucocorticoids and experimental diabetes. Biochem. J. 229, 499–504. https://doi.org/10.1042/bj2290499.

Savitz, J., 2020. The kynurenine pathway: a finger in every pie. Mol. Psychiatr. 25, 131–147. https://doi.org/10.1038/s41380-019-0414-4.

Shaw, W.R., Catteruccia, F., 2019. Vector biology meets disease control: using basic research to fight vector-borne diseases. Nat. Microbiol. 4, 20–34. https://doi.org/10.1038/s41564-018-0214-7.

Sterkel, M., Oliveira, J.H.M., Bottino-Rojas, V., Paiva-Silva, G.O., Oliveira, P.L., 2017. The dose makes the poison: nutritional overload determines the life traits of blood-feeding arthropods. Trends Parasitol. 33, 633–644. https://doi.org/10.1016/j.pt.2017.04.008.

Stone, T.W., Stoy, N., Darlington, L.G., 2013. An expanding range of targets for kynurenine metabolites of tryptophan. Trends Pharmacol. Sci. 34, 136–143. https://doi.org/10.1016/j.tips.2012.09.006.

Summers, K.M., Howells, A.J., Pyliotis, N.A., 1982. Biology of eye pigmentation in insects. In: Advances in Insect Physiology, pp. 119–166. https://doi.org/10.1016/S0065-2806(08)60153-8.

Terenius, O., Lindh, J.M., Eriksson-Gonzales, K., Bussière, L., Laugen, A.T., Bergquist, H., Titanji, K., Faye, I., 2012. Midgut bacterial dynamics in *Aedes aegypti*. FEMS Microbiol. Ecol. 80, 556–565. https://doi.org/10.1111/j.1574-6941.2012.01317.x.

Valach, M., 2016. RNA extraction using the "home-made". Trizol substitute. https://doi.org/10.17504/protocols.io.eiebcbe.

Waage, J.K., 1979. The evolution of insect/vertebrate associations. Biol. J. Linn. Soc. 12, 187–224. https://doi.org/10.1111/j.1095-8312.1979.tb00055.x.

Wang, G.H., Gamez, S., Raban, R.R., Marshall, J.M., Alphey, L., Li, M., Rasgon, J.L., Akbari, O.S., 2021. Combating mosquito-borne diseases using genetic control technologies. Nat. Commun. 12, 1–12. https://doi.org/10.1038/s41467-021-24654-z.

WHO, 2017. Global Vector Control Response 2017–2030.

Wilson, A.L., Courtenay, O., Kelly-Hope, L.A., Scott, T.W., Takken, W., Torr, S.J., Lindsay, S.W., 2020. The importance of vector control for the control and elimination of vector-borne diseases. PLoS Neglected Trop. Dis. 14 (1), e0007831. https://doi.org/10.1371/journal.pntd.0007831.

Yamamoto, D.S., Sumitani, M., Hatakeyama, M., Matsuoka, H., 2018. Malaria infectivity of xanthurenic acid-deficient anopheline mosquitoes produced by TALEN-mediated targeted mutagenesis. Transgenic Res. 27, 1–10. https://doi.org/10.1007/s11248-018-0057-2.

Yuen, T., Zhang, W., Ebersole, B.J., Sealfon, S.C., 2002. Monitoring G-protein-coupled receptor signaling with DNA microarrays and real-time polymerase chain reaction. In: Methods in Enzymology, pp. 556–569. https://doi.org/10.1016/S0076-6879(02)45047-1.