Discovery of knock-down resistance in the major malaria vector *Anopheles funestus* reveals the legacy of persistent DDT pollution.

Authors

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Abstract

A major mechanism of insecticide resistance in arthropod pests is knock-down resistance (*kdr*) caused by mutations in the voltage-gated sodium channel (*Vgsc*) gene. Common in most malaria *Anopheles* vector species, *kdr* mutations have never been observed in *Anopheles funestus*, the principal vector in Eastern and Southern Africa. From whole-genome sequencing of 333 *An. funestus* samples from a breadth of populations in Tanzania, we found 8 novel amino acid substitutions in the *Vgsc* gene, including the *kdr* variant, L1014F (L976F in *An. funestus*), in tight linkage disequilibrium with another (P1842S). The mutants were found only at high frequency in one region, with a significant decline between 2017 and 2023. When evaluating the resistance phenotype of these samples, we found a strong association between L976F and survivorship to the exposure to DDT insecticide, but no association with a pyrethroid insecticide (deltamethrin). No DDT products are currently prequalified by WHO for vector control, and the chemical is banned in Tanzania. However, widespread DDT contamination and a legacy of extensive stockpiles in the same region where we found the *kdr* alleles, may have selected for this evolution. Continued monitoring is necessary to confirm the origin of *kdr* in *An. funestus*, and how it may threaten the effectiveness of insecticide-based vector control in Africa.

Main

Chemical insecticides are central to the control of agricultural pests and disease vectors. The control of *Anopheles* mosquitoes through the distribution of over 2.9 billion insecticide-treated bed nets (ITNs)[1] has helped avert an estimated 633 million cases of malaria[2] - a disease that still kills 600,000 yearly[1]. However, the widespread use of insecticides for agricultural pest and disease vector control also has detrimental consequences, including direct lethal and sub-lethal effects on human and animal

52 health[3,4] and destabilizing effects on ecosystem structure and function. For example,
53 insecticide exposure is a key stressor affecting the population decline of pollinators,
54 essential for ecosystem health and food production[5,6]. These impacts are often
55 unpredictable[7].

56 Strong selection pressures generated by insecticide-based agricultural pest and
57 disease vector control activities have resulted in the independent evolution of a
58 diverse range of mechanisms that confer insecticide resistance (IR) phenotypes in
59 numerous insect species[8]. One of the earliest described IR mechanisms was the
60 emergence of knock-down resistance (*kdr*), mediated by mutations in the target-site of
61 pyrethroid and organochlorine insecticides, located in the voltage-gated sodium
62 channel gene (*Vgsc*), an essential component of the nervous system[9]. These *kdr*-driven
63 resistance phenotypes appeared rapidly after the introduction of the organochlorine
64 dichloro-diphenyl-trichloroethane (DDT) spraying for insect control in the mid-20th
65 century[10]. Knock-down resistance [11,12], the key ingredient in ITNs - the first line of
66 defence against malaria. The threat posed by IR to malaria vector control has led to
67 intense efforts to develop coordinated strategies for IR management that integrate
68 entomological, molecular, and genomic surveillance data towards proactive strategies
69 for mitigating the impacts of IR[13,14].

70 As part of a genomic survey done in Tanzania to understand the evolution and
71 spread of insecticide resistance in *Anopheles funestus* - the dominant malaria vector in
72 Eastern and Southern Africa[15], we report the first discovery of *kdr* mutations in *An.*
73 *funestus.* Despite a complete ban on DDT use for agriculture and vector control in
74 Tanzania since 2008[16], we discover that this mutation confers resistance to DDT, and
75 suggests environmental contamination from extensive DDT stockpiles[17], or unofficial
76 agricultural use, as possible causes. The emergence of *kdr*, which confers resistance to
77 insecticides in major crop pests and vectors of disease (such as *An. gambiae* and *Aedes*
78 *aegypti*)[18], highlights the potential of chemical insecticide contamination or unofficial
79 use to exert unexpected and potentially harmful impacts on public health.

We analysed WGS data from 333 mosquitoes sampled from 10 sites across Tanzania (**Figure 1A**). We performed genome-wide selection scans (GWSS) with the G123 statistic[26] to test for evidence of selective sweeps in the *An. funestus* genome associated with known or novel IR loci (**Figure 1B**); grouping samples by administrative region (see **Supp. Table 1** for per-group sample numbers). We detected a clear signal of elevated G123 in the region containing the *Vgsc* gene in samples from the Morogoro region in the southeastern part of the country (**Figure 1B**). In Kagera, Katavi, and Mwanza regions, there was no visible sign of a selective sweep at or near the *Vgsc* region. In Dodoma, Lindi, Ruvuma, and Tanga, there were peaks of elevated G123 near to *Vgsc*, but these appeared within the context of relatively high G123 across the chromosome **(Figure 1B).**

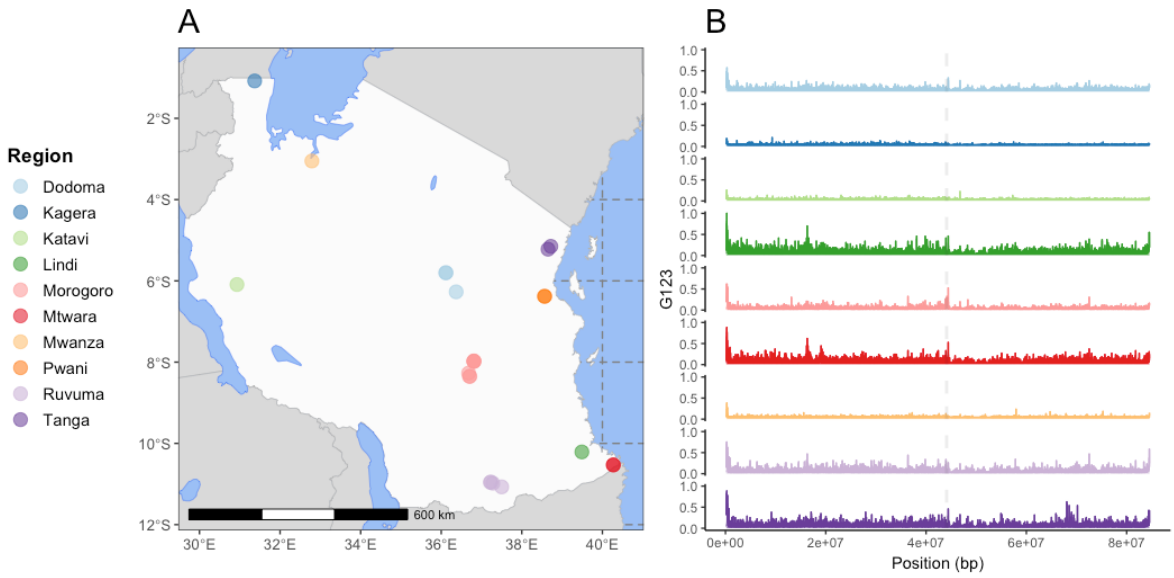


Figure 1: (A) Map of *An. funestus* collection locations. Points indicate sample collection locations. Point colour indicates the administrative region from which samples were collected. (**B**) G123 selection scans of *An. funestus* chromosome 3RL, coloured and windowed by sample collection region (where n>20 – see **Supp Table 2**). X axis indicates position (in base-pairs (bp)), Y axis indicates the selection statistic G123. Grey dotted line indicates the location of the *Vgsc* gene. Note Mwanza region is absent from (**B**) as there were too few samples (n<20) to perform a selection scan.

99

100 Mutations in *Vgsc* confer *kdr* in numerous pest and insect taxa[19]. While not
101 previously reported in *An. funestus*[20], *kdr* mutations in major malaria vectors within
102 the *An. gambiae* complex are subject to intense selection[21,22] and confer resistance to
103 pyrethroid and organochlorine insecticides used in ITNs and insecticide sprays[12,19].
104 We searched our data for mutations in the *Vgsc* gene and found 8 amino acid
105 substitutions occurring at frequencies greater than 5% (**Figure 2A**). Of these, two
106 alleles, L976F and P1842S occurred at the highest frequency (**Figure 2A**). The
107 frequencies of P1824S and L976F were highest in samples collected from Morogoro in
108 2017 (0.75 and 0.90 respectively) **(Figure 2A)** and declined yearly, reaching their
109 lowest frequency in samples collected in 2023 (0.48 and 0.56 respectively; $\chi^2 = 12.15$,
110 p=0.0005; **Figure 2B).** These mutations occurred at very low frequencies or were absent
111 in all other locations (**Figure 2A).** We aligned the *An. funestus Vgsc* sequence (Gene ID:
112 AFUN2_008728) with that of *Musca domestica* (Gene ID: X96668) and *An. gambiae*
113 (AGAP004707-RD AgamP4.12 gene set)[22]. The amino acid change at *An. funestus* L976F
114 corresponded to L1014F in *M. domestica* and L995F in *An. gambiae* in domain II subunit
115 6 (IIS6) of the *Vgsc* gene (**Table 1**), which in *An. gambiae* species complex drastically
116 increases IR to DDT and pyrethroids[12,23]. The second variant P1842S corresponded to
117 P1874S in *An. gambiae* and P1879 in *M. domestica* and were all in the C-terminal domain
118 (**Table 1**).

119 **Table 1**: Comparative non-synonymous nucleotide variation in the voltage-gated sodium
120 channel gene

Variant					Population allele frequency (%)							
Position[a]	**Af[b]**	**Ag[c]**	**Md[d]**	**Domain**	**Dodoma 2022**	**Morogoro 2017**	**Morogoro 2019**	**Morogoro 2021**	**Morogoro 2023**	**Pwani 2019**	**Ruvuma 2019**	**Ruvuma 2022**
3RL:44,115,564 T>C	I2030V	I2061	P2063	COOH	0.00	0.00	0.00	0.06	0.02	0.00	0.00	0.02

3RL:44,115,768 C>A	G1962C	A1994	P1997	COOH	0.00	0.00	0.00	0.00	0.00	0.05	0.06	0.05
3RL:44,116,128 G>A	P1842S	P1874S	P1879	COOH	0.04	0.75	0.69	0.67	0.48	0.10	0.11	0.11
3RL:44,116,334 T>C	N1773S	N1805	N1810		0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00
3RL:44,116,923 A>T	F1638Y	F1670	V1675		0.00	0.05	0.00	0.00	0.04	0.15	0.00	0.00
3RL:44,117,167 A>T	W1557R	W1589	W1594		0.00	0.10	0.14	0.00	0.04	0.00	0.00	0.00
3RL:44,122,391 C>A	G1144C	G1173	G1180		0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00
3RL:44,125,475 T>A	L976F	L995F	L1014	IIS6	0.04	0.90	0.83	0.67	0.56	0.25	0.11	0.11

121 **Position**[a] Position relative to the *Anopheles funestus* strain FUMOZ reference, chromosome arm 3RL.

122 **AF**[b] Codon numbering according to *Anopheles funestus* transcript LOC125769886_t4.

123 **Ag**[c] Codon numbering according to *Anopheles gambiae* transcript AGAP004707-RD in geneset
124 AgamP4.12.

125 Md[d] Codon numbering according to *Musca domestica* EMBL accession X96668 Williamson *et al.*[24].

126

127 In *An. gambiae*, multiple *kdr* haplotypes have evolved independently[25,26]. To
128 elucidate *Vgsc* haplotype structure in *An. funestus,* we computed pairwise linkage
129 disequilibrium (LD) using the Rogers and Huff method[27], between nonsynonymous
130 variants occurring at a frequency of > 5% in Tanzanian *An. funestus* **[Figure 2C**]. We
131 found that P1824S occurred in tight LD with L976F (*D′*=0.95) **[Figure 2C].** Of other
132 non-synonymous polymorphisms, F1638Y and W1557R exhibited only weak LD with
133 L976F [**Figure 2C]**. We constructed a haplotype clustering dendrogram from
134 haplotypes in all 333 individuals, from the *Vgsc* gene **[Figure 3].** The clustering
135 dendrogram disclosed three major clades and three main combinations of the four

136 most prevalent *Vgsc* alleles **[Figure 3].** The most striking signal was a subclade of
137 identical, or near-identical haplotypes containing both L976F and P1842S [**Figure 3],**
138 indicating a selective sweep on a combined L976F/P1842S haplotype. This combined
139 haplotype was present at higher frequencies in the Morogoro region relative to the
140 neighbouring regions of Pwani, Ruvuma, and Dodoma [**Figure 3**]. Most amino acid
141 substitutions were present in a single clade in samples from Pwani, Dodoma,
142 Ruvuma, and especially Morogoro [**Figure 3**]. This extremely restricted geographic
143 hotspot of *kdr* is in stark contrast to its distribution in *An. gambiae* where *kdr* mutations
144 spread rapidly across vast spatial scales in response to strong selection pressure[21,28].

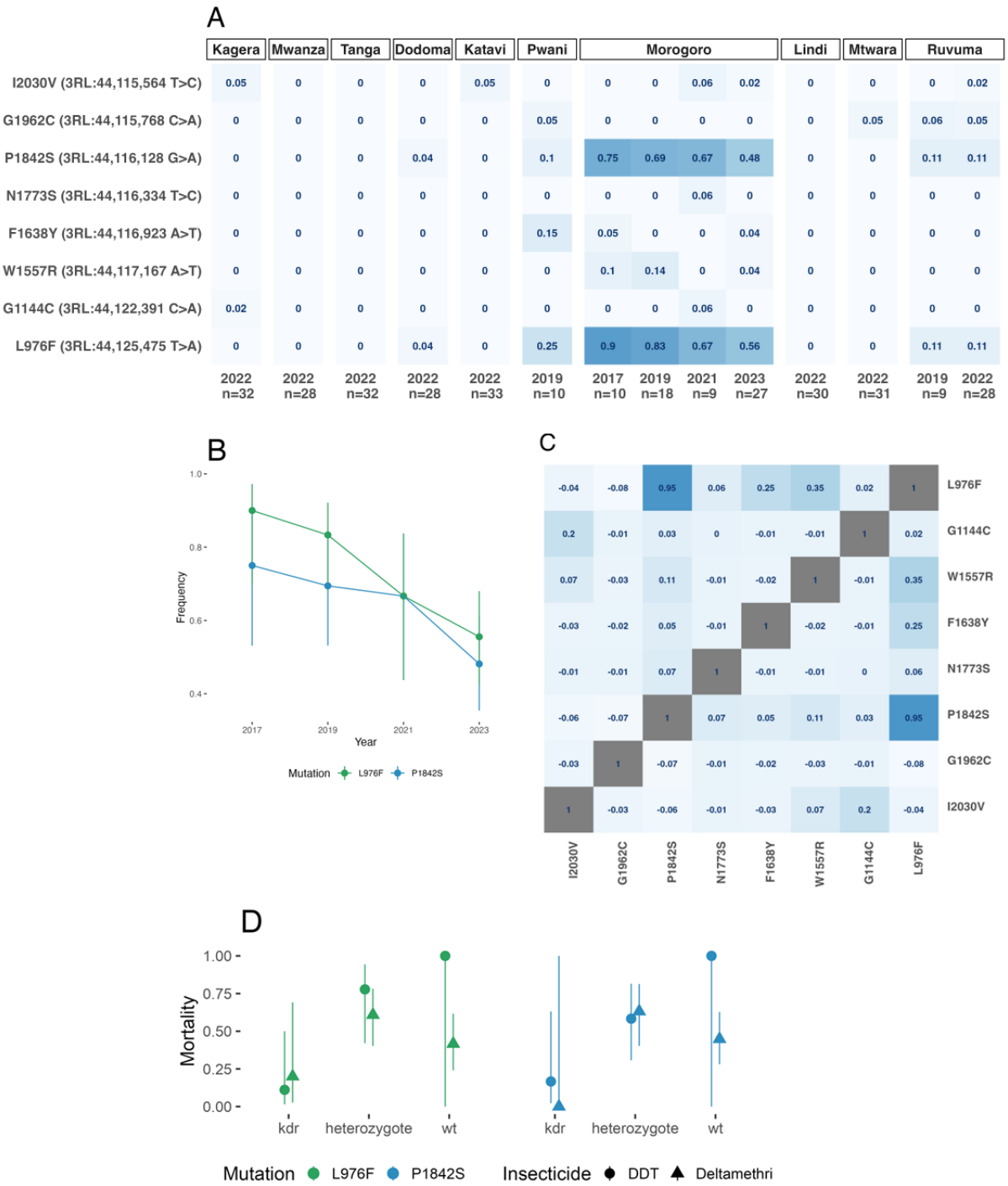
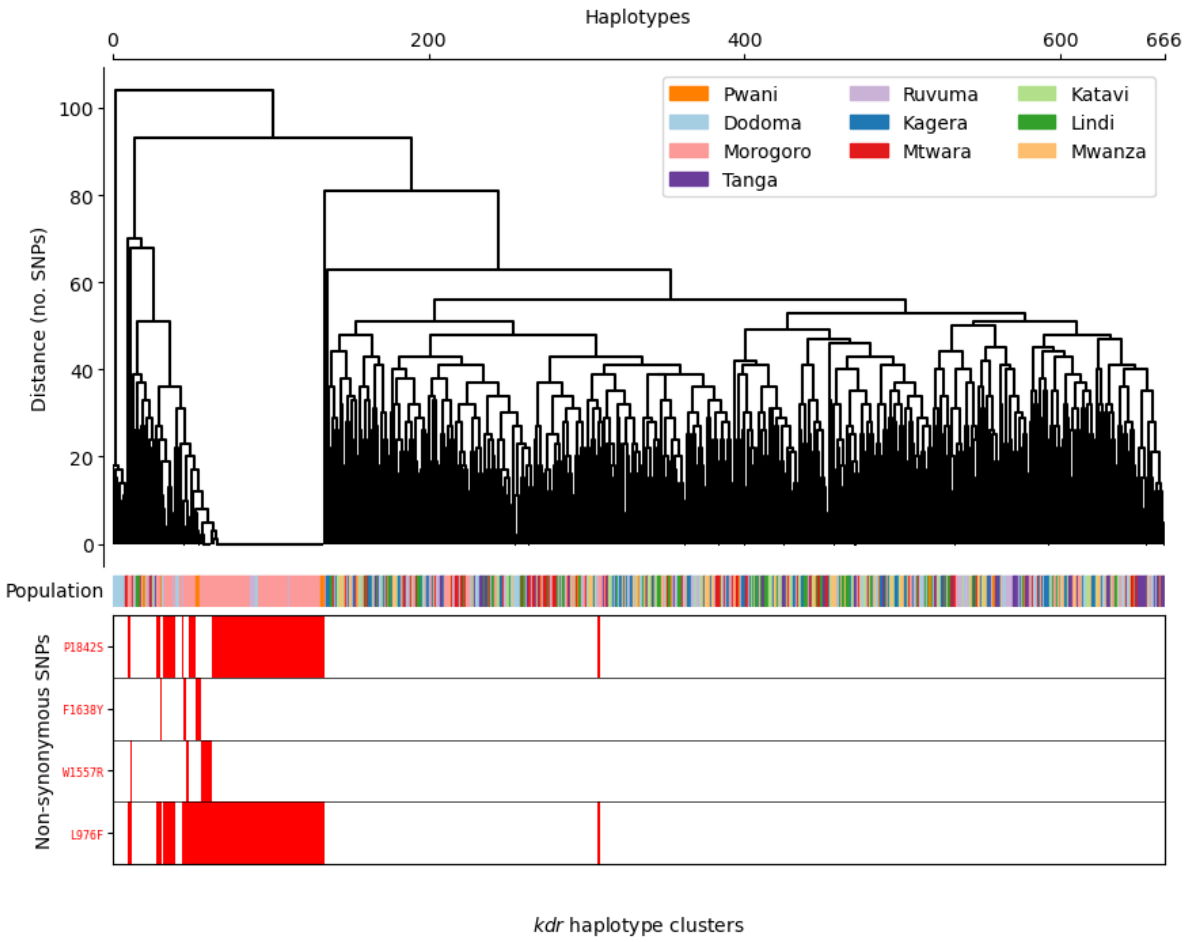


Figure 2: (A) Heatmap of *Vgsc* allele frequencies. Y axis labels indicate mutation effect, chromosome position, and nucleotide change. X axis labels indicate collection date, heatmap intensity indicates frequency where darker = higher, with frequency labelled in each heatmap facet. Heatmap is panelled by sample collection region. (**B)** L976F and 1842S frequencies, in Morogoro region, over time. Y axis indicates allele frequency, X axis indicates date. Line and point colour refer to mutation, specified in the legend. Bars indicate 95% confidence intervals. (**C)** Heatmap of linkage disequilibrium (LD) (Rogers and Huff R)

153 between nonsynonymous variants in the *Vgsc* gene at frequency > 5%. LD is indicated by fill
154 colour. SNP effects and positions are labelled on X and Y axes. (**D**) Denotes the association of
155 L976F and P1842S with resistance to Deltamethrin and DDT. Colour and panelling are by
156 mutation, x axis indicates genotype, y axis indicates mortality, point shape indicates mean
157 for each insecticide and line indicates the 95% CI based on generalised mixed model
158 prediction.



159

160 **Figure 3:** Clustering of haplotypes at the *Vgsc* gene (LOC125769886, 3RL:44105643-44156624).
161 Dendrogram branch length corresponds to no. SNPs difference (y axis). Tips correspond to
162 individual haplotypes (x axis). The coloured Population bar denotes the administrative region
163 of origin (as described by the legend). Red blocks at the bottom indicate the presence of the
164 labelled non-synonymous SNPs in the *Vgsc* gene.

165

166 Resistance to all major classes of insecticide is common in *An. funestus* and is
167 primarily mediated through the increased activity of enzymes that bind to and
168 metabolise insecticides (metabolic resistance)[29,30]. This contrasts with *An. gambiae*
169 where resistance is mostly conferred by a combination of metabolic and target-site
170 resistance[8]. We investigated phenotypic resistance (as measured by mosquito survival
171 following insecticide exposure) in *An. funestus* to the discriminating doses of
172 deltamethrin (type II pyrethroid) and DDT and tested whether the metabolic enzyme
173 synergist piperonyl butoxide (PBO), which is increasingly used on ITNs[31], could
174 restore susceptibility in pyrethroid-resistant populations in Morogoro region. The
175 mosquitoes were phenotypically resistant to both deltamethrin (24-hr mortality: 46%,
176 CI 35.6 – 57.1) and DDT (68%, CI 57.8 - 77.9) but deltamethrin susceptibility was
177 significantly restored in the presence of PBO (95%, CI 85.6 - 98.4). To explore the
178 possible association between L976F and P1842S alleles with DDT and deltamethrin
179 resistance, we genotyped surviving (resistant) and dead (susceptible) mosquitoes
180 from IR bioassays for both L976Fand P1842S loci). Neither locus was associated with
181 deltamethrin resistance: L976F (χ^2 = 0.04, p = 0.84) and P1842S (χ^2 = 0.59, p = 0.44).
182 (**Figure 2D**). We found a strong association with DDT resistance in mosquitoes
183 carrying the mutant allele of L976F (χ^2 = 9.23, odds ratio = 11.0, p = 0.0024) and a
184 marginally non-significant positive association for P1842S (χ^2 = 3.75, p = 0.0528)
185 (**Figure 2D**). DDT is a largely obsolete, banned, pesticide that is no longer widely used
186 for vector control in Tanzania, or in Africa as a whole, due to its bio-accumulative and
187 toxic properties. However, the presence of DDT resistance phenotypes in *An. funestus*
188 in Morogoro in 2015[32] suggests *kdr*-mediated resistance to DDT has been present at
189 least since then, and the emergence of *kdr* resistance to DDT suggests that future use
190 of DDT for IRS may become even less favoured. The lack of association of *kdr* with
191 pyrethroid resistance might be due to the strong metabolic resistance shown to
192 pyrethroids in *An. funestus,* reducing the benefit of *kdr* [33]**.** However, since our testing
193 included only type II pyrethroids, additional data are required to assess potential
194 impacts of *kdr* on other pyrethroids and on commercial products. The association of

195 *kdr* with resistance to DDT but not pyrethroids, combined with selection signals and
196 recently declining *kdr* allele frequencies where we have time series, suggests recent-
197 past, rather than contemporary selection, perhaps due to factors other than the current
198 use of public health pesticides.

199 There is no record of DDT use in the last decade for agriculture or vector control
200 in the Morogoro region, or Tanzania as a whole. Tanzania, as a signatory to the 2009
201 Stockholm Convention on Persistent Organic Pollutants, banned the production,
202 importation, and usage of DDT[16], except for limited use in malaria vector control. DDT
203 was last used for malaria control in indoor residual spraying (IRS) in 2010[34]. The Africa
204 Stockpiles Programme (ASP) was launched in 2005 to eliminate stockpiles of obsolete
205 pesticides, including DDT. At this time, it was estimated that Tanzania still possessed
206 approx. 1,500 tonnes of obsolete pesticides [35]. Intriguingly, Mahenge-Ulanga in the
207 Morogoro region held a DDT stockpile of 4.5 tons (as of 2004) in [36], approximately 100
208 km away from where DDT-resistant *An. funestus* were detected in this study.
209 Additionally, a large stockpile of 19 tons of DDT was also held in Morogoro town,
210 where over 300 tonnes of DDT-contaminated soil were removed for disposal in 2012
211 [17]. The ASP and the Tanzanian Government eliminated 100% of inventoried publicly
212 held DDT stockpiles and conducted extensive environmental remediation by
213 programme close in 2013 [37]. However, extensive DDT contamination remains [38]. For
214 example, 1040 tonnes of DDT contaminated soil were removed in 2014/2015 [36], and
215 DDT remains in use by private individuals. The coincident proximal location of high
216 levels of *kdr* in *An. funestus* with large past DDT stockpiles as well as the presence of
217 widespread DDT contamination and private usage, leads us to hypothesise that the
218 evolution of *kdr* in *An. funestus* may have been driven by DDT contamination of local
219 larval breeding sites. The removal of DDT stockpiles by the ASP, and ongoing
220 environmental remediation, may have contributed to reduced selection pressure on
221 *kdr*, evident from declining frequency in Morogoro. Continued monitoring of allele

frequency and future studies of *kdr* frequencies targeted towards sites of known DDT contamination will establish whether this hypothesis is correct.

Our discovery of *kdr* resistance mutations in the *Vgsc* gene in *An. funestus* is a significant and unwelcome development that has the potential to pose a new threat to vector control in the region. Reassuringly, it seems that the emergence of *kdr* is not linked to, nor is presently likely to threaten, the mass rollout of PBO-pyrethroid bednets currently underway in Tanzania as a response to IR. This does not preclude a role for *kdr* in the *An. funestus* IR arsenal in the future, and the threat posed by *kdr* to vector control in *An. gambiae* means the presence of *kdr* in *An. funestus,* even if it evolved due to exposure to persistent organic pollution, requires close monitoring.

In *Silent Spring* (1962), Rachel Carson brought for the first time into the public eye the unpredictable and often remote impacts of anti-insect chemical agents on human health and on nature "on one hand delicate and destructible, on the other miraculously tough and resilient, and capable of striking back in unexpected ways" [39]. Our findings underscore the legacy of *Silent Spring* by reinforcing the potential for pesticides and organic pollutants to exert unexpected influences on animal biology. If the link we draw between *kdr* evolution and the gradual elimination of DDT and DDT-contaminated material proves definitive, it provides an optimistic outlook for the successful recovery of animal species, and the environment more broadly, in response to human efforts.

Materials and Methods

All scripts and Jupyter Notebooks used to analyse genotype and haplotype data, and produce figures and tables are available from the GitHub repository: https://github.com/tristanpwdennis/kdr_funestus_report_2023

Mosquito collection

248 *Anopheles funestus* samples analyzed in this study were collected from ten
249 administrative regions in Tanzania: Dodoma, Kagera, Katavi, Lindi, Morogoro,
250 Mtwara, Mwanza, Pwani, Ruvuma, and Tanga (**Figure. 1A**). The collections were
251 done as part of a countrywide *Anopheles funestus* surveillance project in Tanzania
252 and were subsequently incorporated into the MalariaGEN *Anopheles*
253 *funestus* genomic surveillance project database
254 (https://www.malariagen.net/projects/anopheles-funestus-genomic-surveillance-
255 project). Mosquitoes were collected in households between 2017 and 2023 using
256 CDC light traps and mechanical aspirators. They were sorted by sex and taxa and
257 *An. funestus* group mosquitoes preserved individually in 96-well plates containing
258 80% ethanol.

259 **Whole genome sequencing and analysis**

260 The samples were processed as part of the *Anopheles funestus* genomics surveillance
261 MalariaGEN Vector Observatory (VObs) project
262 (https://www.malariagen.net/mosquito).Briefly, the mosquitoes were individually
263 whole-genome-sequenced on an Illumina NovaSeq 6000s instrument. Reads were
264 aligned to the *An. funestus* reference genome AfunGA1 [40] with Burrows-Wheeler
265 Aligner (BWA) version v0.7.15. Indel realignment was performed using Genome
266 Analysis Toolkit (GATK) version 3.7-0 RealignerTargetCreator and IndelRealigner.
267 Single nucleotide polymorphisms were called using GATK version 3.7-0
268 UnifiedGenotyper. Genotypes were called for each sample independently, in
269 genotyping mode, given all possible alleles at all genomic sites where the reference
270 base was not "N".

271 Complete specifications of the alignment and genotyping pipelines are available
272 from the malariagen/pipelines GitHub repository
273 (https://github.com/malariagen/pipelines/). The aligned sequences in BAM format
274 were stored in the European Nucleotide Archive (ENA).

275 The identification of high-quality SNPs and haplotypes were conducted using BWA
276 version 0.7.15 and GATK version 3.7-0. Quality control involved removal of samples
277 with low mean coverage, removing cross-contaminated samples, running PCA to
278 identify and remove population outliers, and sex confirmation by calling the sex of
279 all samples based on the modal coverage ratio between the X chromosome and the
280 autosomal chromosome arm 3R. Full quality control methods are available on the
281 MalariaGEN vector data user guide (https://malariagen.github.io/vector-
282 data/ag3/methods.html).

283 We used decision-tree filters that identify genomic sites where SNP calling and
284 genotyping is likely to be less reliable. More information on site filters can be found
285 on the MalariaGEN vector data user guide.

286 Genotypes at biallelic SNPs that passed the decision-tree site filtering process were
287 phased into haplotypes using a combination of read-backed and statistical phasing.
288 Read-backed phasing was performed for each sample using WhatsHap version 1.0
289 [https://whatshap.readthedocs.io/]. Statistical phasing was then performed using
290 SHAPEIT4 version 4.2.1 [https://odelaneau.github.io/shapeit4/].

291 Complete specifications of the haplotype phasing pipeline are available from the
292 malariagen/pipelines GitHub repository
293 (https://github.com/malariagen/pipelines/tree/master/pipelines/phasing-vector).

294 **Identification of SNPs on *Vgsc***

295 To identify the *An. funestus Vgsc* gene and the variant that confers target-site
296 resistance we performed alignments between the *An. gambiae* VGSC transcript
297 AGAP004707-RD in AgamP4.12 geneset from the Ag1000 phase 3 data resource
298 (https://www.malariagen.net/data/ag1000g-phase3-snp) and AFUN2_008728 from
299 the *An. funestus* AfunF1.3 dataset. We extracted single nucleotide polymorphism
300 (SNPs) altering the amino acid of VGSC protein from the *An. funestus* dataset and
301 computed the allele frequency on the mosquito cohorts defined by the region and

302 year of collection ((See **Supp. Table 1** for per region/year sample numbers)). Under
303 selection pressure various alleles are expected to increase in frequency; we therefore
304 filtered out variant alleles with a frequency lower than 5% resulting in a list of 8
305 variant alleles. Multiple sequence alignments of *An. funestus Vgsc* against *An. gambaie*
306 and *M. domestica* were performed using MEGA v11.013.

307 **Population genetic analyses**

308 We searched for signatures of selective sweeps on the *Vgsc* gene using the *G123*
309 selection statistic [41]**.** G123 selection scans were performed on *An. funestus* genotypes
310 by collection region where sample *n*>20 **[**see **Figure 1A** and **Supp Table 2**] **]** using
311 the *g123_gwss* function in the malariagen_data python API
312 (https://malariagen.github.io/malariagen-data-python/latest/Af1.html). Linkage
313 disequilibrium (Rogers and Huff's R-squared) [27] between the 8 *Vgsc* alleles was
314 calculated using the *rogers_huff_r_between* in scikit-allel
315 (https://zenodo.org/record/4759368). Haplotype clustering was performed by
316 performing hierarchical clustering on a Hamming distance matrix, inferred from
317 phased *An. funestus* haplotypes, using the Scipy library (https://scipy.org/citing-
318 scipy/). Clustering dendrogram, and bar plot of amino acid substitutions, was
319 plotted using the seaborn library [42].

320

321 **Association of L976F and P1842S alleles with insecticide resistance**

322 To test for associations between the identified mutations with IR, we exposed wild
323 non-blood-fed *An. funestus* mosquitoes of unknown ages to standard doses of
324 deltamethrin and DDT insecticides following the WHO tube assays. For each
325 insecticide, we randomly separated phenotypically resistant mosquitoes (i.e., alive 24
326 hours post-exposure) and susceptible (i.e., dead 24 hours post-exposure) and
327 extracted DNA from individual mosquitoes using DNeasy Blood and Tissue kit
328 (Qiagen, Germany). The mosquitoes were identified at the species level using

329 species-specific primers that can distinguish *An. funestus* from the other members of
330 the group [43]. To establish if the two *kdr* variants are associated with insecticide
331 resistance, we designed PCR primers from *An. funestus Vgsc* (Gene ID:
332 LOC125769886) to amplify domain IIS6 (L976F) and C-terminal (P1842S) (see **Supp**
333 **Table 3** for primer and thermocycler conditions). The DNA fragments were
334 separated on a 1% agarose gel, cut, purified using PureLink™ Quick Gel Extraction
335 Kit (Invitrogen), and commercially Sanger sequenced. Collectively, we sequenced 76
336 individual mosquitoes: 56 from deltamethrin and the rest from the DDT bioassays.
337 The frequencies of the wild type and mutant alleles were determined and correlated
338 with phenotypes using generalised linear models in R-software v4.1.1.

339 **Data availability**

340 The sequencing data generated in this study have been deposited in the European
341 Nucleotide Archive (https://www.ebi.ac.uk/ena/browser/home) under study
342 number PRJEB2141.

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Contributions

The project was conceived and supervised by FOO, FB, and DW. Field collection was performed by JOO, IHN, HB, and GM. Laboratory analysis, data acquisition and management, and preparing samples for whole genome sequencing were performed

388 by JOO. Sequence QC, alignments, SNP calling, and haplotype phasing were
389 performed by AHK, JN, CSC, and AM. JOO, BP, and TPWD analyzed the data and
390 generated all figures and tables. The manuscript was drafted by JOO and TPWD and
391 revised by all authors. Throughout the project, all authors have contributed key
392 ideas that have shaped the work and the final paper.

393 **Competing interests**

394 The authors declare no competing interests.

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