 doi:10.20944/preprints202103.0693.v1

Article

2La paracentric chromosomal inversion and overexpressed metabolic genes enhance thermotolerance and pyrethroid resistance in the major malaria vector *Anopheles gambiae*

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Simple Summary: While climate-induced shifts in temperature promotes spread of insects, exacerbating intensity of vector-borne diseases, like malaria, very high temperature exerts deleterious effects in insect vectors, forcing them to evolve/adapt genetically. These genetic adaptations may facilitate insecticide resistance through common genes involved in both processes. Here, the impact of thermal tolerance on pyrethroid resistance in the major malaria vector *Anopheles gambiae* from 4 localities spanning savanna and sub-Sahel of northern Nigeria was investigated. In all the 4 localities *An. coluzzii* and *An. gambiae* were the only malaria vectors found. The populations were highly thermotolerant with ~50% of mosquitoes in 2 sites surviving 44°C. Thermotolerant larvae and adult mosquitoes (survivors of 44°C) were significantly more resistant to permethrin suggesting that prior heat-hardening facilitates insecticide resistance. Thermal tolerance and permethrin resistance were found to be associated with 2La rearrangement (a form of chromosomal inversion). Transcriptional analysis revealed 6 major genes commonly overexpressed in the mosquitoes highly thermotolerant and those resistant to permethrin, suggesting common mechanisms involved in thermotolerance and insecticide resistance. These findings highlight challenges associated with insecticide-based malaria vector control within the context of climate change, stressing the need to take climate variables into account for the choice of control measures.

Abstract: Climate change is impacting the spread/intensity of vector-borne diseases, including malaria, and accelerating evolutionary/adaptive changes in vector species. These changes including chromosomal inversions and overexpression and/or changes in allele frequencies of thermotolerance-associated genes, may facilitate insecticide resistance through pleiotropy. This study investigated the impact of thermotolerance on pyrethroid resistance in four populations of malaria vector *An. gambiae*, from savanna/sub-Sahel of northern Nigeria. *Anopheles coluzzii* and *An. gambiae* were the only malaria vectors found, sympatric in all the sites, with the former species predominant. High thermotolerance was observed, with no mortality at 38°C, and LT_{50} of ~44°C. Significantly high permethrin resistance was observed (mortality <50%) in heat-hardened (44°C) larvae from two sites, BUK and Pantami, compared with control, and heat-hardened adult females from Auyo (mortality = 3.00%±1.20, χ^2 = 5.83, p<0.01) compared with control (12.00%±4.65). The 2La chromosomal inversion was detected at ~50% in larvae and 58% in adult females. Significant association was observed (OR = 7.2, p<0.03) between permethrin resistance and $2La/+^a$ rearrangement compared with $2L+^a/+^a$, in BUK larvae. For all sites permethrin resistance correlated with 2La/a homozygosity in adult females [OR = 5.02, p=0.01). qRT-PCR identified 6 genes commonly induced/overexpressed, including heat shock protein 70 (AGAP004581) which was 2468x and 5x overexpressed in heat-hardened and permethrin-resistant females, respectively, trehalose-6-phosphate synthase (AGAP008227), and ionotropic glutamate receptor genes, *IR25a* (AGAP010272) and *IR21a* (AGAP008511). This study

 doi:10.20944/preprints202103.0693.v1

highlights challenges associated with insecticide-based malaria vector control, and the epidemiological significance of taking climate variables into account for design/choice of control measures.

Keywords: malaria; *Anopheles; coluzzii*; *gambiae*; 2La; chromosomal; inversion; thermotolerance; permethrin; resistance

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1. Introduction

Global warming driven by emissions from humans of the Anthropocene Age [1] is speeding up climate change and the magnitude of its impact on humans and natural systems [2]. The increase in global mean surface temperature (GMST) of 1.5°C in the 21st Century is projected to increase the risk of vector-borne diseases [2]. Weather and climate are among the drivers of *Anopheles* geographic range, intensity of transmission and seasonality of malaria, with burden of the disease projected to increase with climate change because of a greater geographic range of the *Anopheles* vector, shifts in phenology [3], increase in number of generations per year [4], longer season, and/or increase in the number of people at risk [5-7]. The population of mosquito vectors are projected to shift, but with contrasting expansions and reductions depending on the degree of local warming and the ecology of the mosquito vectors [5], leading to regionally variable patterns [2,7].

Small ectotherms (such as mosquitoes) are constrained by the extrinsic thermal environments, from microclimates to regional climates [8], and are particularly sensitive to even daily temperature fluctuations [9]. Indeed, temperature has been shown to constrain mosquito development rate, egg to adult survival and mortality rate, modifying biting rate and fecundity, regulating *Plasmodium falciparum* parasite development rate and vector competence in the two major malaria vectors *Anopheles gambiae* [10] and *Anopheles stephensi* [11]. Empirical evidence from *An. stephensi* suggest a temperature optimum for transmission of 26°C (minimum and maximum of 17°C and 35°C, respectively). Increasing environmental temperature during the larval stages has been established to impact thermal performance. For example, in *An. gambiae* s.s. increase of 4°C [(e.g. from 23°C to 27°C, etc), 8°C (27°C to 35°C) and 12°C (23°C to 35°C) significantly increased larval mortality [12], while increase of 8°C significantly lowered adult survival)]. With respect to vector control, ambient temperature impact the efficacy of many public health insecticides e.g. *An. gambiae* and *An. stephensi* [13], having a marked effect on the toxicity of the most used insecticides for malaria control, stressing the need to evaluate the efficacy of insecticides/control tools under real field conditions [14]. Lower temperature (below laboratory standard of 26°C) has been shown to increase susceptibility of *An. stephensi* to malathion, with little impact on permethrin susceptibility [15]. Increase in temperature has been established to enhance deltamethrin resistance of a susceptible population of *An. arabiensis*, while exposure at temperatures both lower and higher than standard insectary conditions increased mortality in susceptible *An. funestus* and resistant *An. arabiensis* females [14].

Climate change (increased aridity and drylands) is accelerating evolutionary/adaptive changes in animal species, including insect pests and disease vectors [4,16], potentially selecting evolutionary winners, with the losers, e.g. the species lacking adaptive capacity living near physiological limits and prone to extinction [4]. These adaptive, genetic changes occur at chromosomal levels [for example, chromosomal inversions [17]] and/or changes in the allele frequencies of genes involved in thermotolerance and desiccation resistance [16], with increasing periods of thermal stress and drought predicted to produce directional selection for insecticide resistance [4].

Paracentric chromosomal inversions are one of the most effective instruments for speciation and local adaptations [17-19], which are maintained in spatially and temporally heterogenous environment, segregate along climatic gradients of aridity (https://www3.nd.edu/~nbesansk/Inversions_2018.html) and their frequencies is known to be strongly and significantly correlated with a number of adaptive, phenotypic traits in *Anopheles*. The 2La inversion in *An. gambiae* s.l. strongly correlates with degree of aridity across environmental gradients [20,21], increasing northward from humid to the arid regions in West/Central Africa [20,22], suggesting that the 2La arrangement confers a selective advantage in xeric habitats, while the alternative $2L+^{a}$ arrangement is more beneficial in mesic habitats [20]. The *An. gambiae* s.l. carrying $2L+^{a}$ allele have been shown to be more susceptible to *Plasmodium falciparum* infection [23]. *An. gambiae* carrying 2La allele are also associated with resistance to desiccation in adults [24,25] and thermal stress in larvae

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[20,26], were less prone to rest indoors [21] and it was shown that inversion 2La assorts with insecticide resistance, e.g. dieldrin plus fipronil [27].

Several studies have investigated the genetic basis of thermal stress and desiccation resistance. These include measurement of cuticle thickness and cuticular hydrocarbons (CHC) composition in 2La and 2L+^{a} karyotypes which linked 2La arrangement with thicker cuticle and differences in the CHC composition associated with lower rate of water loss for the 2L+^{a} karyotype [28]. Comparative gene expression profiling of heat hardened 2La and 2L+^{a} larvae have established a common and massive induction of a core set of heat-shock genes, with the 2La allele preconditioned with a much more aggressive response with larger numbers of upregulated genes, which are heat responsive and involved in proteolytic degradation and energy metabolism [20]. Fine-scale association mapping of desiccation tolerance within the 2La and the 2Rb inversions of *An. gambiae* has revealed dozens of significant single nucleotide polymorphisms within both 2La and 2Rb inversions, many of which neighboured genes controlling ion channels or related functions, with transcriptional profiles strongly influenced by karyotype and genes inside rearranged regions overrepresented among those differentially expressed genome-wide [29]. Two of the top-ranking candidate genes discovered in the above recent study have prominent roles in response to environmental stimulus: AGAP006026 encodes an ionotropic glutamate receptor (IR) commonly associated with chemosensation, thermosensation and hygrosensation [30,31] and AGAP006961 [32] encodes a heat-shock protein (*hsp90*).

While several studies have address the impact of 2La inversion on adaptation and insecticide resistance [17,27], little is known on the impact of this inversion on pyrethroid (major ingredients in bed nets) resistance in the field population of *An. gambiae*. Also, the few genome-wide transcriptional and association studies carried out to identify the major genes involved in thermotolerance, have not investigated the pleiotropic action of the discovered genes on insecticide resistance. This constitute a knowledge gap hampering insecticides resistance management in field populations of the major malaria vectors, within the context of globally warming world.

This study investigated the thermal tolerance breadth of in *An. gambiae* s.l. (*An. gambiae s.s.* and *An. coluzzii*) in four sites spanning the northern Guinea savanna and Sudan/sub-Sahel of northern Nigeria, and the role of chromosomal inversion and metabolic resistance genes in tolerance to heat stress and insecticide resistance. Thermotolerance and insecticide resistance were found correlated with 2La inversion polymorphism, in heterozygote (2La/+^{a}) and homozygote (2La/a) forms, respectively. qRT-PCR transcriptional profiling of heat-hardened and permethrin-resistant *An. coluzzii* established induction/overexpression of core set of heat shock proteins previously associated with heat stress, as well as other common heat- and pyrethroid-resistance associated genes, including 2 ionotropic receptors and a trehalose phosphate synthase/phosphatase.

2. Materials and Methods

2.1. Sampling sites and mosquito populations

To capture heterogeneities in malaria vector compositions and/or various chromosomal forms of Anopheles collection was carried out in 4 sites spanning Guinea, Sudan, and sub-Sahel savanna of northern Nigeria (Figure S1). Larvae collection was preferred in place of indoor-resting blood fed females to avoid bias from collection of the most endophilic species [33,34]. Collections were conducted in the rainy months of July, August and September, 2019 (mid-July through August, to mid-September) in temporary rain puddles in (i) Bayero University Kano (BUK), situated in Sudan savanna of Kano City (11°58′17″N, 8°35′9″E); (ii) Gamjin Bappa, a Sudan savanna village in Karaye, Kano State (11°46'23.6"N, 8°00'29.9"E); (iii) Pantami, a northern Guinea savanna town located in Gombe State (10°15'50.4"N, 11°09'39.7"E), and (iv) in irrigation rice paddies in Hadiyau, a

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village in the sub-Sahel of Auyo, Jigawa State (12°21′38″N, 9°59′15″E). Collections were done using classical dipping method [35] and larvae maintained under standard insectarium condition (~70-80% relative humidity and 25-27°C), supplemented with Tetramin™ Baby fish food, with 12:12h day/night cycle.

2.2. Morphological and molecular identification of mosquito larvae and adults

For every collection larvae were identified as belonging to the *Anopheles gambiae* Complex using morphological keys [36] and maintained as described above. When required larvae were reared to adulthood, and adults maintained as above, with 10% sucrose solution. A subset of larvae and adults which were used for thermotolerance and insecticide resistance bioassays (see below for details) were homogenised and DNA extracted using LIVAK method [37]. SINE200 PCR [38] was used to identify the larvae and adults to species level.

2.3. Initial assessment of thermotolerance profile using temperature gradient

Initial thermotolerance profiling of *Anopheles* species was conducted by exposing L4 larvae to various temperature gradients (5°C-46°C) for 1h, using a modified protocol of Rocca [26]. Larvae from BUK and Hadiyau were subject to 11 different temperature treatments (5°C, 7°C, 10°C, 15°C, 27°C, 38°C, 42°C, 43°C, 44°C, 45°C and 46°C), while those from Gamjin Bappa and Pantami were subjected to 6 temperature regimens: 38°C, 42°C, 43°C, 44°C, 45°C and 46°C only (due to sample size). 8 replicates each of 25 larvae were used for the lower temperatures (5°C through to 27°C), while 4 replicates of 25 larvae were used for the higher temperatures (38°C through to 46°C (due to sample size). The L4 larvae were placed in 50ml glass tubes containing 20ml of deionised water, pre-set in a water bath at the experimental temperatures, and allowed to remain for 1h. Tubes were cooled by transferring into water bath set to 27°C and larvae fed with Tetramin food. Number of larvae dead at 24h was recorded. In each assay, control groups received same treatment, except that they were maintained at ambient temperature. 4 replicates of 25 L4 larvae of *An. coluzzii* (Ngoussou) were exposed to 43°C and 44°C, as well, to assess thermotolerance status of a known, fully insecticide susceptible colony domesticated in insectary. Ngoussou larvae were procured from the LITE, at LSTM, United Kingdom (https://lite.lstmed.ac.uk/).

2.4. Impact of heat hardening on pyrethroid resistance

To investigate the effect of short-term thermal stress on pyrethroid resistance, larvae were pre-exposed for 30min at 44°C, with resting for 12h at 27°C, followed by dose-response bioassays with 12.5mg/mL, 25, 50 and 100mg/mL of permethrin. For each concentration, 4 replicates of 20-25 larvae were utilised in experiments which were conducted using the WHO procedure [39], with mortalities scored after 24h. For each of the 4 concentrations 4 replicates of 20-25 unexposed larvae were used as controls (exposed to respective concentrations of permethrin, but not 44°C). Negative controls were also set for each treatment arms above and were (i) exposed to water containing the solvent (methanol) used to dissolve permethrin, and (ii) maintained in water alone.

To investigate impact of long-term heat-hardening on resistance, another set of the larvae which survived 44°C were reared to adulthood and 2-5 day-old females used for WHO tube bioassays with permethrin [40]. 4 replicates of 20-25 females were exposed to impregnated papers containing discriminating doses of permethrin (0.75%) for 1h, transferred to holding tubes and supplied with 10% sucrose. Mortality was recorded 24h after exposure. Two controls were used: (i) 2 replicates of 20-25 adults which survived 44°C but not exposed to insecticide; and (ii) 2 replicates of 20-25 adults unexposed to 44°C and unexposed to insecticide.

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2.5. Molecular karyotyping of 2La and 2L+^{a} inversion polymorphism

To establish correlation between thermotolerance and inversion polymorphism larvae were genotyped for 2La polymorphism. These include 44°C survivors (thermotolerant, T_R) and those that died (T_S). Also, alive (Insecticide resistant, I_R) and dead (I_S) larvae from 100mg/ml permethrin exposure were also genotyped, to correlate insecticide resistance at immature stage with the inversion. In addition, adult females, permethrin-alive and permethrin-dead from WHO tube bioassays were also genotyped to establish correlation between pyrethroid resistance and inversion, in adult stage. These were adults raised from larvae not exposed to any heat stress (not heat-hardened). All larvae used for genotyping were identified to species level, by DNA extraction with the LIVAK protocol, followed by SINE200 PCR (described above).

Molecular karyotyping was carried out using PCR [41,42] with primers 23A2 (Universal reverse), 27A2 (for 2La) and DPCross5 (for 2L+^{a}). PCR mix comprised 11.85µL of ddH_20, 5µL of 5X Buffer, 25mM $MgCl_2$ (2µL), 2.5mM dNTP mix (2µL), 1µM (1µL) each of the above 3 primers and 5U/µL of GoTaq DNA polymerase (Promega, Wisconsin, USA). Thermocycling conditions were 94°C for 2min, followed by 35 cycles each of 94°C for 30s, 60°C for 30s and 72°C for 45s; and a final extension at 72°C for 5min. PCR amplicons were separated on 2% agarose gel stained with pEqGREEN and visualised for bands. Product sizes for the 2La and 2L+^{a} arrangements were 492bp and 207bp, respectively, with heterozygotes having both bands.

2.6. Transcriptional profiling of thermotolerance-related genes using qRT-PCR

To investigate the potential role of pleiotropic genes on thermotolerance and insecticide resistance, 3-4 day-old females which survived exposure at 44°C for 30min (and allowed to rest for 12h), those which survived exposure to 0.75% permethrin and unexposed females were used for qRT-PCR, targeting 9 genes previously associated with thermotolerance and/or insecticide resistance [20,31,43-46]. These include 6 heat shock proteins: [*hsp90* molecular chaperone HtpG (AGAP006959), *hsp90* beta (AGAP001424), *hsp83* (AGAP006958), *hsp70* 1/8 (AGAP004944), *hsp90* ATPase activator (AGAP010514) and *hsp70* (AGAP004581)]; trehalose-6-phosphate synthase/phosphatase (*TPS 1/2*, AGAP008227), and 2 ionotropic receptor genes, *IR21a* (AGAP008511) and *IR25a* (AGAP010272). Primers utilised for the qPCR are provided Supplementary Table 1.

qRT-PCR was carried out using cDNA extracted from 1µg of total RNA from three biological replicates each of the female survivors of heat hardening at 44°C (HH_R), females unexposed to temperature stress, Control (UNX_C), females which survived exposure to 0.75% permethrin (Insecticide resistant, I_R) and unexposed females from the fully insecticide susceptible laboratory colony, Ngoussou. Protocol followed was as done previously [47], with relative expression level and fold change (FC) of each gene in exposed and control females relative to susceptible calculated according to the $2^{-\Delta\Delta CT}$ method, incorporating the PCR efficiency [48], after normalization with the housekeeping genes ribosomal protein S7, *RSP7* (AGAP010592) and *elongation factor Tu* (AGAP005128).

2.7. Data Analysis

R version 3.6.1 (https://cran.r-project.org/bin/windows/base/) was utilized to calculate Odds Ratio (epiR package) for the relationship between thermotolerance and permethrin resistance with 2La inversion polymorphism. LT_{50} and LC_{50} were calculated and dose-response plots created with generalised linear model (glm) using the MASS package. Plots of results of larval and adult bioassays were done using the GraphPad Prism version 7.02 (GraphPad Inc., La Jolla, CA, USA). Statistical analyses of were carried out using a two-tailed Chi-Square test of independence as implemented in GraphPad

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Prism. Data from larvae and adults 2La genotyping were also analysed using multiple correspondence analyses with FactoMineR (for analysis) and factoextra (ggplot2-based visualisation) packages of R, to establish correlation between phenotype and genotype.

3. Results

3.1. Distribution and composition of Anopheles gambiae species

The larvae found in all 4 sites, in collections spanning 3 months were *An. coluzzii* and *An. gambiae* s.s. However, these species varied in their composition, with *An. coluzzii* predominant in Auyo and BUK (File S3). From subset of the larvae exposed to 44°C, 98 were PCR-identified [22 from Auyo (19 *An. coluzzii* and 4 *An. gambiae* s.s.), 27 from BUK (25 *An. coluzzii* and 2 *An. gambiae*), 19 from Gamjin Bappa (8 *An. coluzzii* and 11 *An. gambiae* s.s.) and 30 from Pantami (18 *An. coluzzii* and 12 *An. gambiae* s.s.)]. *Anopheles coluzzii* constituted 70.4% (69) of total larvae used for this bioassay, with *An. gambiae* s.s. constituting 29.6% (29). From subset of the larvae exposed to 100mg/mL permethrin, 84 were PCR-identified [17 from Auyo (14 *An. coluzzii* and 3 *An. gambiae* s.s.), 20 from BUK (18 *An. coluzzii* and 2 *An. gambiae* s.s.), 17 from Gamjin Bappa (10 *An. coluzzii* and 7 *An. gambiae* s.s.) and 30 from Pantami (16 *An. coluzzii* and 14 *An. gambiae* s.s.)]. *Anopheles coluzzii* constituted 69% (58) and *An. gambiae* s.s. 31% (26). From sub-set of adults used for bioassays with 0.75% permethrin 114 were PCR-identified [28 from Auyo (25 *An. coluzzii* and 3 *An. gambiae* s.s.), 32 from BUK (31 *An. coluzzii* and 1 *An. gambiae* s.s.), 28 from Gamjin Bappa (17 *An. coluzzii* and 11 *An. gambiae* s.s.) and 26 from Pantami (10 *An. coluzzii* and 16 *An. gambiae* s.s.)]. *Anopheles coluzzii* constituted 72.8% (83) and *An. gambiae* s.s. 27.2% (31).

3.2. Thermotolerance profile and its intensity in Anopheles gambiae larvae

Initial exposure to various temperature revealed a high thermotolerance with no mortality at all in the larvae which were subjected to lower temperature series (5°C-38°C) (File S1). For Auyo populations mortalities of 1, 9, 42, 90 and 99% were obtained at 42, 43, 44, 45 and 46°C respectively, resulting in estimated LT_{50} (lethal temperature that killed 50% of larvae) of 44.09°C (95% CI: 43.96 – 44.22) (Figure 1). Similar pattern was observed in BUK population with no mortality at 42°C, but mortalities of 3.5, 38, 90 and 100% at 43, 44, 45 and 46°C, respectively (LT_{50} = 44.19°C, CI: 44.08 – 44.31). No mortality was also obtained at 42°C with larvae from Gamjin Bappa, but at 43, 44, 44 and 46°C mortality increased to 1.25, 47.5, 95 and 96.25%, respectively (LT_{50} = 44.10°C, CI: 43.97 – 44.23). For Pantami no mortality was seen at 42 and 43°C, but at 44, 45 and 46°C, 42.5, 66.25 and 100% of the larvae died (LT_{50} = 44.44°C, CI: 44.29 – 44.58). Highest mortality was observed in Ngoussou, at 37% for 43°C and 100% for 44°C, suggesting that the laboratory colony is more thermo-susceptible than the field populations.

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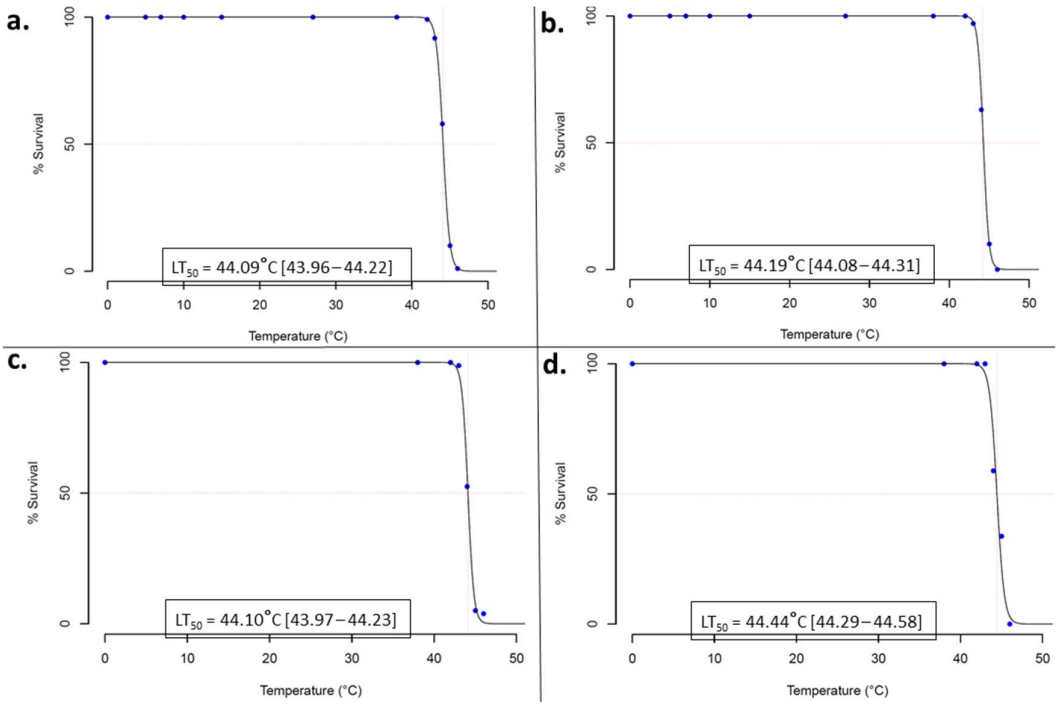

Figure 1: Thermotolerance profile of various populations of *Anopheles gambiae* larvae. Dose-response (temperature-course) bioassays for **a.** Auyo, **b.** BUK, **c.** Gamjin Bappa and **D.** Pantami. LT_{50}, the temperature that killed 50% of the larvae are given as inset with 95% confidence intervals in brackets.

3.3. Impact of heat hardening on pyrethroid resistance

Bioassay using the 44°C heat-hardened larvae revealed a high permethrin resistance in all 4 populations, with mortalities of <50% on average for even 100mg/mL (Figure 2, File S2). Mortalities followed dose-dependent pattern with lowest obtained from 12.5mg/mL permethrin. Significant differences in mortalities were observed between experimental and control larvae, for BUK (50mg/mL, $\chi^2 = 3.908$, $p<0.05$ and 12.5mg/mL, $\chi^2 = 6.25$, $p<0.01$) and Pantami (12.5mg/mL, $\chi^2 = 5.76$, $p<0.01$) (Figure 2b and -d, respectively). The LC_{50} (concentration of permethrin that killed 50% of the larvae) were high (in ranges of 102–184mg/mL), with Gamjin Bappa exhibiting the highest LC_{50}, and specifically the experimental larvae of this population exhibiting higher LC_{50} compared with the control.

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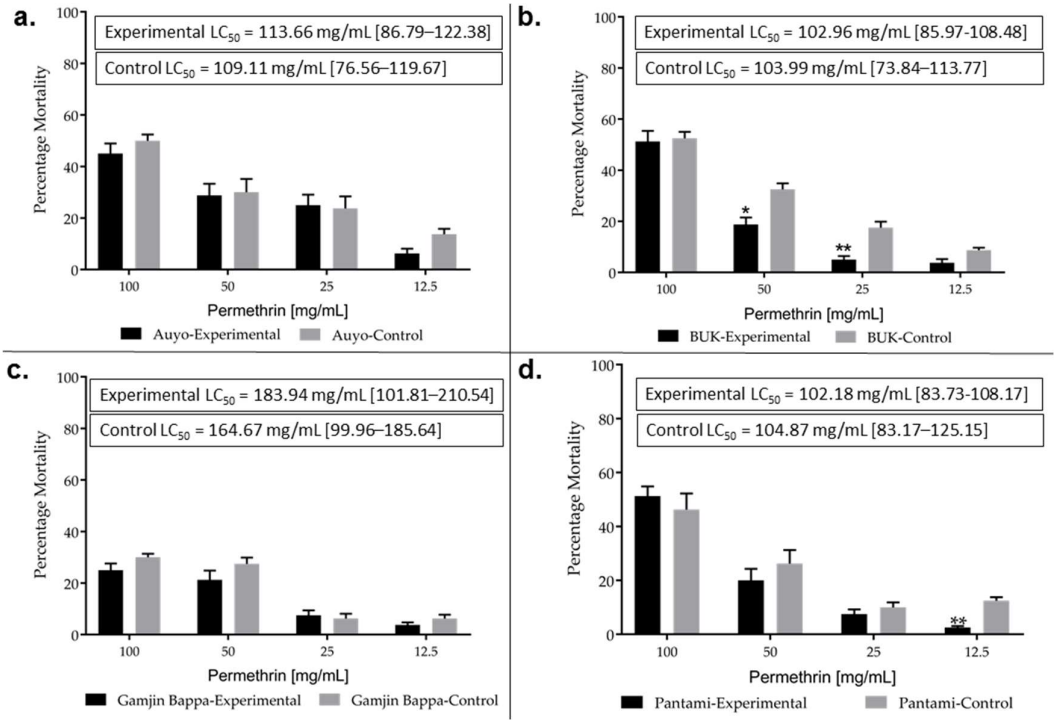

Figure 2: Resistance profiles of *Anopheles gambiae* larvae. Results of WHO larval bioassay with permethrin, for **a.** Auyo, **b.** BUK, **c.** Gamjin Bappa and **d.** Pantami. * and ** = statistically significant at $p<0.05$ and $p<0.01$, respectively from two tailed χ^2 square test of independence. LC_{50} for experimental and control larvae for each of the 4 sites are provided in rectangular inset with 95% confidence limits.

Adult bioassay with permethrin revealed high resistance, with mortalities of less than 20% in both experimental and control cohorts. However, differences in mortalities were observed between the experimental cohorts (raised from larvae which survived exposure at 44°C) and control (Figure 3). For example, significantly lower mortality ($\chi^2 = 5.83$, $p<0.01$) was obtained from Auyo experimental cohort (3.00% ± 1.20) compared with control (12.00% ± 4.65). For the other 3 sites mortalities were not significantly different between experimental and control, though in all cases it was higher in the control.

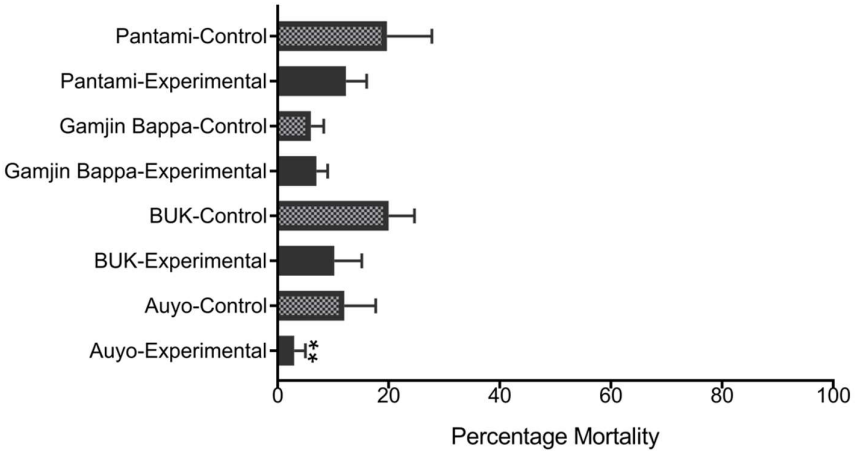

Figure 3. Permethrin susceptibility profiling of heat-hardened and control adult *An. gambiae* females. WHO bioassays with 0.75% permethrin. ** = significantly different from the control at $p<0.01$.

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3.4. Role of 2La chromosomal inversion on thermotolerance and pyrethroid resistance

To investigate the role of chromosomal inversion on thermal stress and permethrin resistance, larvae used for thermotolerance tests (alive and dead from 44°C exposure), and those exposed to 100mg/mL permethrin were karyotyped for 2La inversion (Figure S2 and S3, respectively for agarose gel pictures). Also, adults exposed to 0.75% permethrin (both alive and dead) were genotyped for the inversion (Figure S4).

For BUK larvae exposed to 44°C, significant association [Odds Ratio, OR = 7.2 (1.08 - 4.79), χ^2 = 4.68, p<0.03) was observed between survival and heterozygosity (2La/+^{a} arrangement) compared with 2L+^{a}/+^{a} form (Table S2). For the other 3 sites the differences were not significant, as well as for the combined data. Overall, from the larvae successfully genotyped, 55 (56.12%) were alive and 43 (43.88) dead, and the distribution of 2La/a, 2La/+^{a} and 2L+^{a}/+^{a} were 16 (13.33%), 36 (36.73%) and 46 (46.94%), respectively. Multiple correspondence analysis (MCA) identified variables that explained highest variability in the data. The eigenvalues (which determine number of variables to consider) revealed dimensions 1 - 5 as constituting 81.8% in the variability in the data [Scree plot in Figure S5a showed contribution of the dimensions, and S5b and S5c describe the variable contributions and cos2 (inertia of the variables), respectively]. Variable categories with similar profiles clustered together on the factor map, for example, Pantami, Gamjin Bappa and *An. gambiae* s.s. clustered on the positive quadrant of dimension 1 consistent with the high percentage of this species in these sites (Figure 4a). The alive and 2La/+^{a} clustered together on the negative quadrant of dimension 2 supporting the significant correlation observed between 2La/+^{a} and survival of heat stress. Auyo, BUK, *An. coluzzii* and 2L+^{a}/+^{a} clustered together since this species is the dominant one in these two sites, and since 2L+^{a}/+^{a} arrangement is not linked to thermal and desiccation tolerance, an alternative mechanism is possibly responsible for thermotolerance in *An. coluzzii* (note that the distance between alive and *An. coluzzii* indicates their similarity compared to the variable dead, which cluster with 2La/a arrangement). The variables *An. coluzzii* and *An. gambiae* s.s. which clustered on the opposite side of dimension 2 pole contributed the most to the inertia of the factor map, followed by 2La/+^{a} inversion arrangement (Figure 4b).

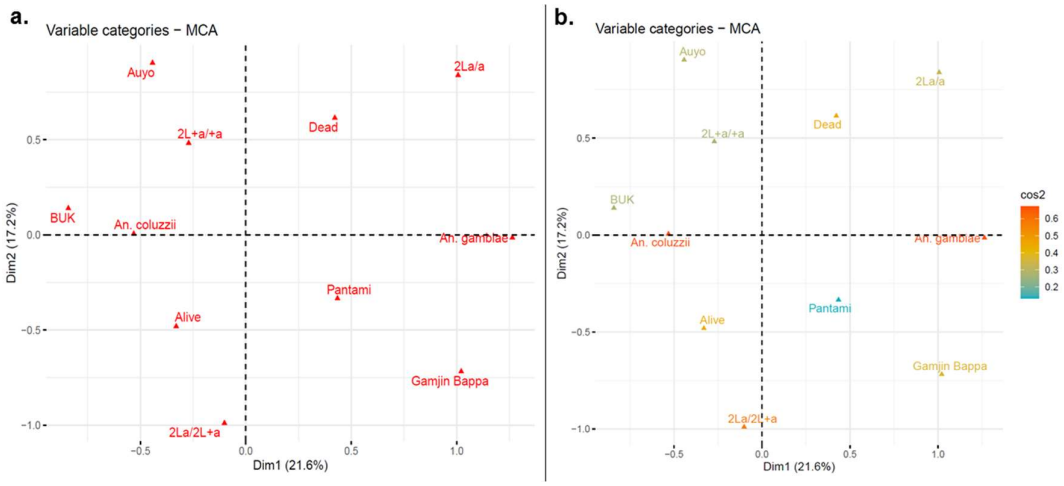

Figure 4: MCA plot of variables from 44°C heat stress bioassay with larvae. a. asymmetric/biplot variables on dimensions 1 and 2. Variable categories with similar profiles are grouped together. Negatively corelated variable categories are positioned on the opposite side of the plot origin (opposed quadrants). Variables that cluster close to the pole, positive or negative of each dimension indicates how important is their contribution to that dimension; **b**. cos2 of the variables with *An. coluzzii* and *An. gambiae* s.s. (*An. gambiae* in the plot) exhibiting the highest inertia/contribution.

For larval bioassay with permethrin no significant difference in distribution of 2La inversion was observed between alive and dead in each site and for the combined data. From

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the larvae successfully genotyped, 41 were resistant (48.8% alive) and 43 were dead (51.2%), and the distribution of 2La/a, 2La/+^{a} and 2L+^{a}/+^{a} were 15 (17.85%), 32 (38.09%) and 37 (44.04%), respectively.

In contrast, for the adult bioassays there was low frequency of 2La/a arrangement among the dead females, with none in BUK and Gamjin Bappa. A significant association [OR = 5.02 (1.48 - 6.93), χ^2 = 7.45, p=0.01) was observed between survival and 2La/a homozygosity when alive and dead females were compared, for all data. Overall, from the adults successfully genotyped 83 (72.8%) were alive and 31 were dead (overall percentage mortality = 27.2), and the distribution of 2La/a, 2La/+^{a} and 2L+^{a}/+^{a} were 25 (21.92%), 42 (36.84%) and 47 (41.23%), respectively. The eigenvalues revealed dimensions 1 - 5 as contributing 84.8% in the variability of the data [Scree plot in Figure S5d shows contribution of dimensions, and S5e and S5f demonstrated the variable contributions and cos2 (inertia of the variables), respectively]. Variables 2L+^{a}/+^{a} and dead clustered together (Figure 5a); Auyo, BUK, *An. coluzzii* and 2La/+^{a} clustered together, consistent with the high percentage of this species in these sites; alive and 2La/a were most close in the right quadrant of dimension 1 supporting the observation of significant association between permethrin survival and the inversion in homozygous state. As in the thermotolerance bioassay the variable category, species (*An. coluzzii* and *An. gambiae* s.s.) contributed the most inertia for the dimensions (Figure 5b).

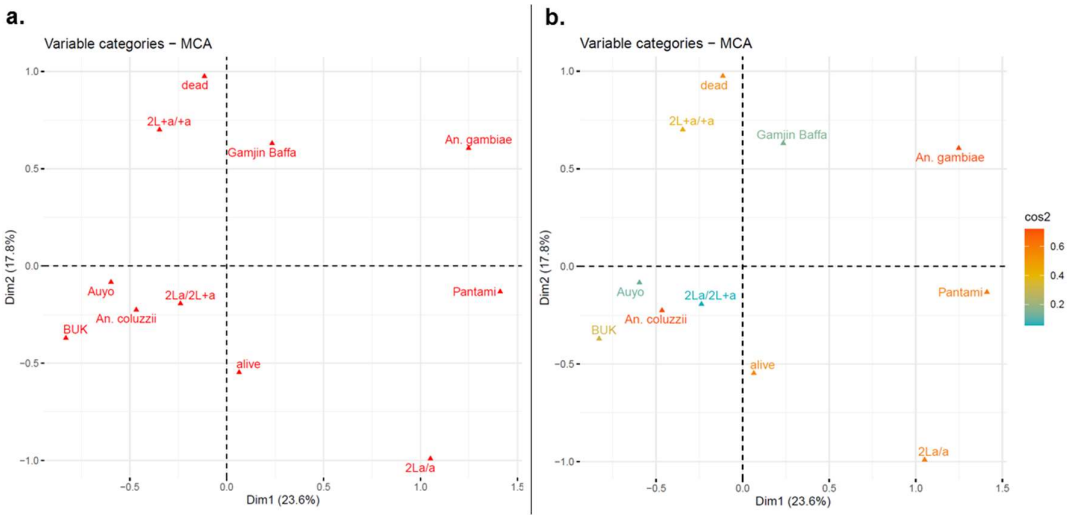

Figure 5: MCA plot of variables from adult permethrin bioassay. a. asymmetric/biplot of variables on dimensions 1 and 2. Variable categories with similar profiles are grouped together. Negatively corelated variable categories are positioned on the opposite side of the plot origin (opposed quadrants). Variables cluster close to the pole, positive or negative of each dimension indicates how important is their contribution to that dimension; **b.** cos2 of the variables with *An. coluzzii* and *An. gambiae* s.s. (*An. gambiae* in the plot) exhibiting the highest inertia/contribution

3.5. Impact of heat hardening on expression profile of thermotolerance- and resistance-associated genes

Comparative profiling of expression of 9 genes associated with thermotolerance and/or resistance revealed the common metabolic genes differentially expressed with respect to thermal stress and pyrethroid exposure. Three major heat shock proteins were highly overexpressed in heat-hardened mosquitoes and to some level in permethrin resistant females (Figure 6). The most overexpressed gene was *hsp70* (AGAP004581), massively induced in heat hardened (HHR) females, with fold change (FC) of 2468, FC of 4.64 in permethrin-resistant females (IR), and FC of 1.08 in unexposed females (UNXC), all compared to Ngoussou. This was followed by *hsp83* (AGAP006958) with FC of 125.2, 5.9

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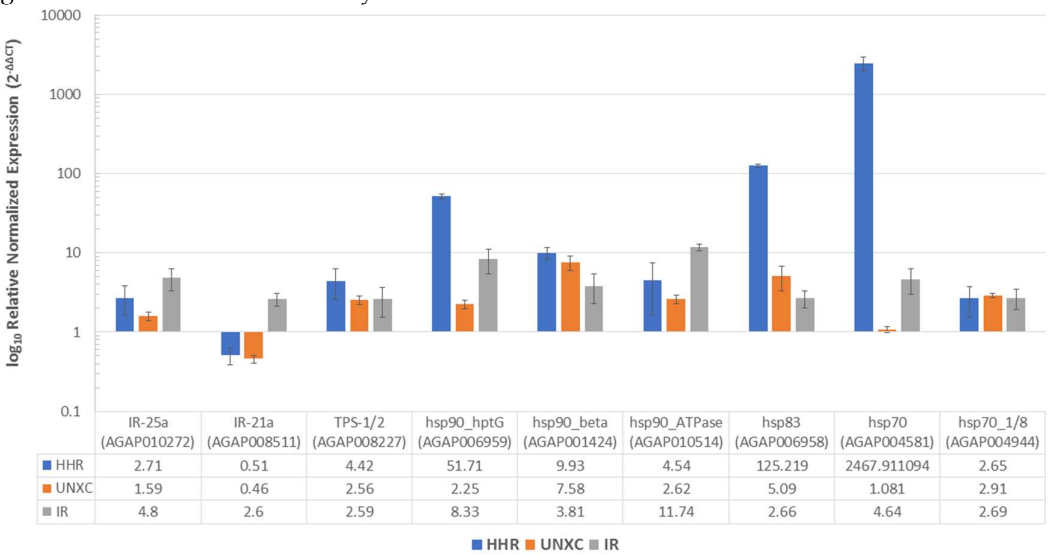
and 2.7 in HHR, UNXC and IR, respectively. The third most overexpressed gene was the *hsp90* hptG (AGAP006959) which was also induced in HHR and IR, with FC of 51.7 and 8.3, respectively. *TPS-1/2* (AGAP0008227) was also induced, with highest FC of 4.42 in HHR, and comparable expression of 2.59 and 2.56 in the IR and UNXC, respectively, highlighting its constitutive overexpression in the field. The 2 ionotropic receptor genes were also differentially expressed with *IR-25a* (AGAP010272) having highest FC of 4.8 in IR, followed by HHR (FC = 2.71) and UNX (1.59). The *IR-21a* (AGAP008511) was only induced significantly in the permethrin resistant (IR) females (FC = 2.6) suggesting the role of this gene in insecticide resistance only.



	IR-25a (AGAP010272)	IR-21a (AGAP008511)	TPS-1/2 (AGAP008227)	hsp90_hptG (AGAP006959)	hsp90_beta (AGAP001424)	hsp90_ATPase (AGAP010514)	hsp83 (AGAP006958)	hsp70 (AGAP004581)	hsp70_1/8 (AGAP004944)
HHR	2.71	0.51	4.42	51.71	9.93	4.54	125.219	2467.911094	2.65
UNXC	1.59	0.46	2.56	2.25	7.58	2.62	5.09	1.081	2.91
IR	4.8	2.6	2.59	8.33	3.81	11.74	2.66	4.64	2.69

Figure 6: Differential expression profiles of 9 genes putatively linked with thermotolerance and/or insecticide resistance. Comparison of heat-hardened adult, female *An. coluzzii* (HHR), Unexposed females (UNXC) and adult survivors of permethrin exposure (IR) to the fully susceptible laboratory colony, Ngoussou. Fold-changes were obtained from the average of 3 independent biological replicates, each of 3 technical replicates. Error bars represent standard deviation. Fold change data table shown below the x axis. Y-axis log to base 10 transformed due to the very high overexpression of *hsp70* in HHR females.

4. Discussion

Under the projection of the future warmed world only mosquitoes equipped with genetic and/or plastic advantages can survive harsh conditions of tropical Africa, particularly the xeric, arid Sahel in West Africa. Chromosomal inversion polymorphism (which promote ecological flexibility and enable exploitation of heterogeneous environments) and/or a suite of pleiotropic genes involved in thermosensation/thermoregulation, hygrosensation and chemosensation conferring adaptive benefit in arid region, as well as protection from insecticide-based vector control measures may select for a DVS in the future. Understanding the role of inversion polymorphism and the genes enhancing life traits in the major malaria vectors will help in reducing malaria risk and promote evidence-based resistance management.

4.1. Evidence of sympatry between An. coluzzii and An. gambiae s.s in northern Nigeria

In this study the two Anopheles mosquitoes, *An. coluzzii* and *An. gambiae* s.s. were the only malaria vector species found in 4 sites spanning northern-Guinea savannah, Sudan savanna and sub-Sahel of northern Nigeria. Despite the sympatric existence of these sibling species no *An. coluzzii*/*An. gambiae* s.s. hybrids were obtained strengthening the evidence of positive assortative mating within these molecular forms [22]. However, in line with our recent findings across Sudan/Sahel [33-35,49,50] *An. coluzzii* was the major

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vector found in all the sites, even though *An. gambiae* s.s. was also in high proportion in the less xeric Gamjin Bappa and Pantami. Previous observations have described *An. arabiensis* as the dominant malaria vector species (DVS) in the northern-most drier/arid environments of Sudan Savannah and Sahel, extending along increasing latitudinal cline, for example, in Niger, Nigeria and Cameroon [22,51-53]. But, within the last few years *An. arabiensis* have become replaced by the pervasive *An. coluzzii* which has become the DVS in the Sahel of southern Niger and central Chad, and in sub-Sahel of northern Nigeria and Cameroon. This is possibly due to this species higher exploitation of breeding sites associated with anthropogenic activities and behavioural plasticity to avoid predators [54], and surviving long dry season *in situ*/aestivation which allows it to predominate and become the primary force of malaria transmission [55,56]. Also, photoperiod and lower nightly temperature have been shown to significantly increase the longevity of *An. coluzzii* - a mechanism which will allow it to diapause in the dry season and re-establish first in the early rainy season [57].

4.2. Evidence of high intensity thermal stress tolerance in Anopheles gambiae larvae

The larvae used in this study exhibited a broad thermal breadth with no mortalities between 5–38°C. The high thermotolerance observed in these populations (LT_{50} of ~44°C) suggests that the larvae are equipped with mechanisms to survive in xeric conditions. A study carried out 2 decades ago have utilised ranges of temperature (37– 44.5°C) to establish in *An. albimanus* larval mortality occurring in a very narrow temperature range, between 40 to 43°C [58], with 100% of larvae dead at 44.5°C. The findings of the present study closely agree with the observations of the above study. Another study on *An. gambiae* have used 40°C to estimate relationship between exposure time and survival rate, showing that mortality increased linearly with time [26]. Also, increased temperature (23 – 35°C for rearing to adulthood) have been shown to decrease *An. gambiae* larvae survival and reduce adult longevity [11].

4.3. Heat hardening enhances insecticides tolerance in larvae and adult Anopheles gambiae

Temperature has been shown to be a critical factor modulating insecticide resistance. In this study short-term exposure at 44°C enhanced permethrin resistance in both larvae and adults *An. gambiae*. A previous study has reported augmentation of pyrethroid resistance in both resistant and susceptible populations of adult *An. arabiensis* exposed to 37 and 39°C [59], with the insecticide resistant population living longer at higher temperature. In contrast, another recent study has found that increasing temperature enhanced the deltamethrin resistance of the susceptible *An. arabiensis* populations while exposure at temperatures both lower and higher than standard insectary conditions increased mortality in resistant population of this species [14]. Other studies have shown a positive temperature coefficient on insecticides, for example in *An. gambiae and An. stephensi* [13], and in other non-Anopheles insects [60].

4.4. 2La chromosomal inversion enhances thermotolerance and permethrin resistance

Chromosomal inversion is known to segregate along climatic gradient of aridity, increasing in frequency along geographical clines, from mesic to xeric environments of the arid regions [21,24]. In this study, frequency of 2La arrangement was found to be on average between 50% in the larvae karyotyped and 58% in the adults, with the heterozygote arrangement ($2La/+^{a}$) on average twice the frequency of homozygote form. 2La homozygotes have been shown to be absent in cyclodiene resistant populations of *An. gambiae* from northern Nigeria [27]. Also, the frequency of 2La inversion in natural populations of *An. gambiae* from Kenya has been shown to decrease from 100% to 17% in less than a decade due to pressure from increased ownership and use of insecticide treated nets which could select against house entering or indoor resting [61], reversing the selection of 2La chromosomal form.

 doi:10.20944/preprints202103.0693.v1

This study established a correlation between 2La inversion polymorphism (heterozygote form, 2La/+^{a} only) and thermotolerance in a single population out of the 4 studied, strengthening previous observations that linked the 2La chromosomal form with ability to survive arid environment. The lack of phenotype-genotype correlation in the other 3 sites could be due to low sample size used for genotyping, and/or differences in the two distribution of the 2 Anopheles species between the 3 sites. However, the finding of high frequency of heterozygote arrangement 2La/+^{a} suggests its possible association with phenotypic advantage, keeping adaptive alleles in heterozygote state, to avoid the otherwise highly deleterious homozygote alleles [62]. Adult *An. gambiae* carrying 2La allele are known to be desiccation resistant [24,25] and heat-hardened larvae carrying the arrangement exhibited thermal stress tolerance higher than alternative arrangement [20,26].

Summing the data from all the 4 sites, the 2La/a arrangement was also found associated with permethrin resistance in adults, when compared to 2L+^{a}/+^{a} . Inversion in chromosome 2 have been shown to modulate insecticide resistance in *Anopheles* mosquitoes. For example, 2Rb inversion was associated with DDT resistance in *An. arabiensis* from Ethiopia [63] and *An. gambiae* from northern Nigeria [64], and 2La/+^{a} heterozygote *An. gambiae* has been shown to be resistant to cyclodienes [27]. The link between inversions in chromosome 2 and insecticide resistance is not surprising since many detoxification genes are located within the inverted regions in this chromosome, for example the voltage-gated sodium channel, *CYP6P3* and *CYP6P4* located within the 2La and 2Rc inversions, respectively [65].

4.5 Common metabolic resistance genes are associated with thermotolerance and permethrin resistance

Short term heat-hardening has been shown to benefit *Drosophila* [66,67] by inducing heat shock proteins [68] which act as molecular chaperones for denatured proteins from heat stress [69]. In this study short-term heat-hardening induced overexpression of several heat shock proteins, most especially the *hsp70* (AGAP004581). This inducible gene has been implicated in thermal acclimation in *Drosophila*, with level of its expression increasing with time of exposure [68] and has been known to be involved in ameliorating the stress of blood-feeding, which is associated with elevated body temperature in *Aedes aegypti* [44]. The fact that this chaperone protein is also overexpressed in permethrin-resistant females suggest its pleiotropic effect toward insecticide resistance. Three other hsp genes found to be overexpressed in both heat-hardened and permethrin-resistant cohort are heat shock proteins *hsp90* (AGAP006959, hptG), AGAP006958 (part of the 3 tandemly arranged *hsp83* genes) and *hsp90* co-chaperone Aha1. These three hsp gene together with the *hsp70* were among the core set of hsp genes involved in a common and immediate response to thermal stress in *An. gambiae* populations [20]. The other two hsp genes found to be overexpressed in the natural populations compared with Ngoussou were *hsp90*_beta and *hsp70* 1/8.

Trehalose (synthesized by *TPS 1/2*) contribute to the maintenance of warm temperature, a condition needed for survival of *Anopheles* [43,70] and is known to promote longevity, fecundity and cold tolerance in insects [46]. *TPS 1/2* was also found to be overexpressed in heat-hardened, unexposed, and permethrin-resistant females, compared with Ngoussou, with the highest expression in heat-hardened females. Ionotrophic receptors have been shown to be a requirement for humidity sensing (hygrosensation), with *IR25a* and *IR21a* known to mediate both humidity and temperature preference in the fruit fly, *Drosophila melanogaster* [31,45], in addition to *IR21a* driving heat seeking and heat-stimulated blood feeding in *An. gambiae* [45]. The level of expression of *IR25a* was found to be higher than *IR21a*, with the highest expression in permethrin-resistant individuals, followed by heat-hardened cohorts. This suggests possible role of these glutamate receptors in thermotolerance and insecticide resistance.

Amongst the genes found induced/overexpressed *hsp70* (AGAP004581), AGAP001424 (*hsp90*), AGAP004944 (*hsp70* 1/8), *TPS 1/2* and *IR25a* are among the set of genes significantly upregulated from recent RNAseq-based genome-wide transcriptional analysis in

pyrethroid resistant populations of *An. coluzzii* from the Sahel (in preparation). Additional work needs to be done to fully elucidate the molecular basis of adaptation to high temperature and its link to insecticide resistance. For example, the role of heat shock proteins in chaperoning the major insecticide resistance genes (using functional genomics and protein biochemistry) can be exploited as Achille's heels to target insecticide resistant mosquitoes.

5. Conclusions

Global warming/climate change associated with increased temperature is impacting the spread and intensity of infectious diseases, through insect vectors. Temperature critically influence the life traits of insects, and its increase lead to more generations per year, increase species range and favour survival of insecticide resistant *Anopheles* vectors, impacting epidemiological effects in terms of malaria transmission. This study has established sympatric existence of the major malaria vectors *An. coluzzii* and *An. gambiae* s.s. in northern Nigeria, has shown that the species are highly thermotolerant and highly resistant to permethrin, in both immature and adult stage. The study also established the role of 2La chromosomal inversion in both thermotolerance (in larvae) and permethrin resistance (in adults) and identified common metabolic resistance genes potentially involved in thermotolerance and permethrin resistance in adult *An. coluzzii*, the DVS from the sites studied. These findings highlight the challenges associated with malaria vector control and provides a glimpse of the mechanisms that mosquitoes are likely to use to adapt to future drastic climate change and the sustained usage of insecticide-based malaria control tools.

Supplementary Materials: The following are available online, Figure S1: **Map of the four sampling sites in northern Nigeria.** Red star for BUK and Gamjin Bappa (Kano State), Auyo (Jigawa State) and Pantami (Gombe State), Figure S2: **Agarose gel of 2La karyotyping using larvae from heat stress bioassay. a.** top panel is for Auyo alive (lanes 1-13) while the lower panel is Auyo dead (lanes 1-9); **b.** BUK alive 1-13; **c.** BUK dead, 1-15; **d.** Gamjin Bappa alive (1-11) and dead (12 and 13); and **e.** Gamjin Bappa dead (1-3), Pantami alive (4-19) and Pantami dead (20-33), Figure S3: **Agarose gel of 2La karyotyping using larvae from permethrin bioassay. a.** BUK alive (lanes 1-9), BUK dead (10-20), Gamjin Bappa alive (21-26) and Gamjin Bappa dead (27-37); **b.** Pantami alive (1-15) and Pantami dead (16-30); **c.** Auyo alive 1-11 and Auyo dead 12-17. Number 18 not identified from species identification**,** Figure S4**: Agarose gel of 2La karyotyping using adult females from permethrin bioassay. a.** Auyo alive (lanes 1-16), Auyo dead (17-28), BUK alive (29-31); **b.** BUK alive (1-19), BUK dead (20-29), Pantami alive (30); **c.** Pantami alive (1-16), Pantami dead (17-26), Gamjin Bappa dead (27-35); and **d.** Gamjin Bappa alive (1-19), Figure S5: **Figure S5: Relative contribution of variables on the factor map. a., b.** and **c.** are Scree plot of the 7 dimensions, cos2 and variables contributions, respectively for dimensions 1-5, from heat stress bioassay. Highest values reflect highest contribution to the dimension (variability); **d., e.** and **f.** are Scree plot of the 7 dimensions, cos2 and variables contributions, respectively for dimensions 1-5, from adult bioassays with permethrin.

Author Contributions: Conceptualization, S.S.I., CSW; methodology, M.M.M., S.S.I., A.M.; validation, S.S.I., C.S.W.; formal analysis, S.S.I.; investigation, M.M.M., S.S.I.; resources, S.S.I., C.S.W.; data curation, S.S.I.; writing—original draft preparation, S.S.I.; writing—review and editing, S.S.I., A.M., M.M.M.; visualization, S.S.I.; supervision, S.S.I.; project administration, S.S.I.; funding acquisition, S.S.I. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Wellcome Trust (https://wellcome.ac.uk/), grant number WT201918/Z/16/Z and the APC was covered by the Wellcome Trust.

Acknowledgments: Not Applicable

Conflicts of Interest: The authors declare no conflict of interest. No funder has a role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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