Parasites & Vectors

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Enrichment of phenotype among biological forms of *Anopheles stephensi* Liston through establishment of isofemale lines

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Abstract

Background Vector management programs rely on knowledge of the biology and genetic make-up of mosquitoes. *Anopheles stephensi* is a major invasive urban malaria vector, distributed throughout the Indian subcontinent and Middle East, and has recently been expanding its range in Africa. With the existence of three biological forms, distinctly identifiable based on the number of ridges on eggs and varying vectorial competence, *An. stephensi* is a perfect species for developing isofemale lines, which can be tested for insecticide susceptibility and vectorial competence of various biological forms.

Methods We describe key steps involved in establishment and validation of isofemale lines. Isofemale colonies were further used for the characterization of insecticide susceptibility and differential vector competence. The results were statistically evaluated through descriptive and inferential statistics using Vassar Stat and Prism GraphPad software packages.

Results Through a meticulous selection process, we overcame an initial inbreeding depression and found no significant morphometric differences in wings and egg size between the parental and respective isofemale lines in later generations. IndCh and IndInt strains showed variations in resistance to different insecticides belonging to all four major classes. We observed a significant change in vectorial competence between the respective isofemale and parental lines.

Conclusions Isofemale lines can be a valuable resource for characterizing and enhancing several genotypic and phenotypic traits. This is the first detailed report of the establishment of two isofemale lines of type and intermediate biological forms in *Anopheles stephensi*. The work encompasses characterization of fitness traits among two lines through a transgenerational study. Furthermore, isofemale colonies were established and used to characterize insecticide susceptibility and vector competence. The study provides valuable insights into differential susceptibility status of the parental and isofemale lines to different insecticides belonging to the same class. Corroborating an earlier hypothesis, we demonstrate the high vector competence of the type form relative to the intermediate form using homozygous

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lines. Using these lines, it is now possible to study host-parasite interactions and identify factors that might be responsible for altered susceptibility and increased vector competence in *An. stephensi* biological forms that would also pave the way for developing better vector management strategies.

Keywords *Anopheles stephensi*, Isofemale, Biological forms, Vectorial competence, Insecticides, Homozygosity, *Plasmodium* susceptibility

Background

The adaptive evolution of mosquitoes (considered as the deadliest animal) dates back to the time of dinosaurs from the Mesozoic to species in the Holocene era [1]. Owing to the diversity of mosquitoes and their rapid rate of evolution [2–4], many research groups across the world work on various aspects of the mosquito vector including its biology, molecular genetics, physiology, population genetics, developmental biology, evolutionary biology, behavior and so on. During these studies, colonization of mosquitoes under laboratory conditions for the purpose of experimentation becomes essential. In recent years, several researchers have developed techniques to rear mosquito species that were previously considered difficult to maintain under laboratory conditions [5, 6]. One such effort led to the colonization of *Anopheles stephensi* under controlled environmental conditions. The rearing protocols with optimal temperature, light and humidity are established for standard insectary operations [7–9]. *An. stephensi* exhibits a greater magnitude of variation across populations, demanding the establishment of isofemale lines with greater genetic homogeneity, enabling the study of the quantitative and qualitative traits under laboratory conditions [10]. The molecular variations at the genomic, transcriptomic and proteomic levels can be better established in isofemale lines [11]. Isofemale lines have been previously used for studying behavior [12], morphometry [13], insecticide resistance [14, 15], pathogen susceptibility and speciation among mosquitoes and other insect species. By enrichment of phenotypes, isofemale lines enable the identification of associated genotypes, which are otherwise diluted in their heterozygous parent lines [16]. Establishing an isofemale line has been shown to delineate the non-additive genetic variance due to local adaptations, bottleneck events and other epistatic effects [17].

Furthermore, when isofemale lines are maintained as isofemale colonies for many generations, they can be used for any live material experimental studies [18]. During the initial stages of colonization, insect populations pass through a genetic bottleneck [19]. Quantitative traits tend to vary greatly because of adaptation to the rearing conditions. However, since insectary operations are well standardized, maintaining the isofemale lines under these regimes would yield reliable the quantitative traits determined from the mean values. Though there are publications related to the establishment of isofemale lines, the techniques and success vary depending on the species (*Drosophila* [17], *Trichogramma* [20], *Chilo* sp. [18] and mosquitoes [13, 21]) depending on their biology. There are also many instances of unsuccessful establishment of isofemale lines due to improper mating, poor reproductive success, inbreeding depression, etc. [22]. Among many hurdles, inbreeding depression is a daunting challenge that can be mitigated with careful line establishment practices and selection of the founder females in the filial generation with key fitness traits [13, 20, 23, 24]. In the present article, we outline a detailed procedure for the establishment of two isofemale lines from insectary colonized populations—a type form "IndCh" derived from TIGS-2 (T2) and an intermediate form "IndInt" derived from TIGS-6 (T6) [25]. We report the characterization of the following parameters: (i) fitness, (ii) homozygosity across filial generations, (iii) insecticide susceptibility and (iv) vectorial competence for both these lines.

Materials and methods

Collection and colonization of mosquitoes

Mosquitoes were collected from their natural habitats through larval or adult sampling as per the previously established protocols [26]. The T6 strain was originally collected from the semi-urban area of Sriramanahalli, Bangalore, Karnataka (12.972442°N, 77.580643°E), at the larval stage in 2016. The T2 strain was collected from the urban area of Anna Nagar, Chennai, Tamil Nadu (13.018410°N, 80.223068°E), at the adult stage in 2016. The rearing was standardized as per previously established protocols [27]. The adults were identified using the identification keys of Nagpal and Sharma [28]. On day 6 or 7, the adults were blood-fed through a standard membrane feeding system (approval ref. no. TIGS 2nd IBSC Oct 2018). For T2 populations, the resting blood-fed adults were collected inside concrete houses. The blood-fed adults were spotted in the corners of the room, resting on dark curtains. Around 60 adults were collected with the help of aspirators between 6 and 8 a.m. during summer. T6 populations were collected in fat larval stages from fresh water in new open cement tanks, primarily used to provide water for cows. Underground bore

well water is used to replenish these tanks on a weekly basis. Around 250 larvae were collected from two nearby tanks.

Establishment of isofemale lines

The isofemale progenies were developed based on a method previously described by Ghosh and Shetty [29]. About 20–25 gravid females (G0) were separated from their parental colonies (T2 and T6) maintained in the TIGS insectary. On day 3, each gravid female was transferred carefully to a single ovicup for egg laying. The individual females were allowed to lay eggs undisturbed for ~24–48 h under laboratory conditions. The single G0 female that laid the most eggs and had the highest percent hatchability was selected for the establishment of isofemale lines. Eggs collected from the single female were checked for egg ridge number and allowed to hatch. Larvae were provided with larval food and reared to adults as described earlier. Emerged adult siblings (G1) were kept in a rearing cage and allowed to inbreed (sibling mating). Around 5–10 mated females were randomly separated for continuing the filial generations. The same protocol was followed in subsequent filial generations (20 generations for IndCh and 23 generations for IndInt)(Fig. 1). Key life table parameters like fecundity (number of eggs produced per female), egg morphology, percent egg hatchability, number of larvae and pupae, sex ratio (male:female), longevity of adult females (lifespan) and frequency of blood meals taken by a female were recorded in every generation to check the fitness of the isofemale lines.

Establishment of isofemale colony

Both IndCh and IndInt isofemale lines were maintained as two separate isofemale colonies after 20 generations and 23 generations, respectively, instead of single female progeny [29]. These two lines are currently being maintained in TIGS insectary. In both the colonies, males and females were allowed to inbreed within the filial generation, blood-fed and allowed to lay eggs in masses. All the key fitness parameters were monitored and recorded. These two isofemale colonies were utilized for insecticide-susceptibility assays and vectorial competence studies with *Plasmodium berghei* and *Plasmodium falciparum*.

Morphometric analyses

Different developmental stages of the mosquitoes were carefully measured for their morphometric characteristics. In the present study, we have measured the eggs and wings in parental and isofemale lines.

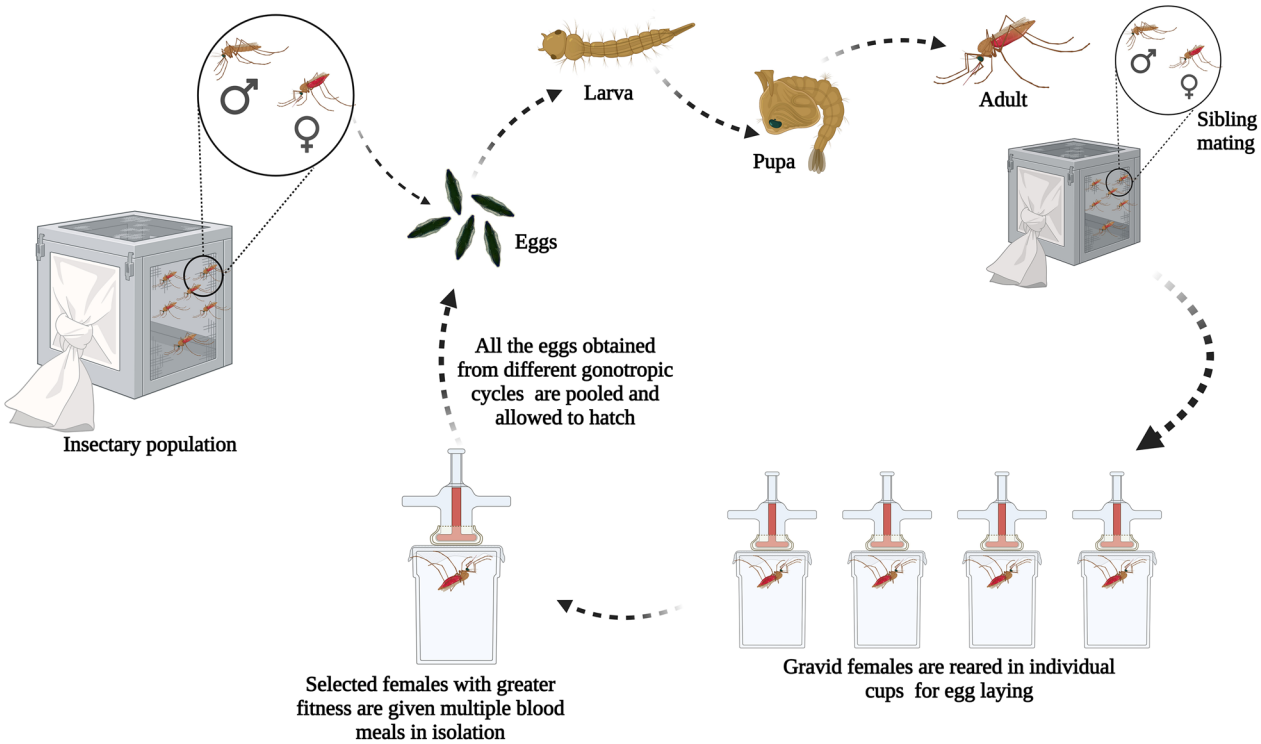

Fig. 1 Schematic representation of the establishment of isofemale lines

Egg parameters

In total, five parameters—egg ridge number, shape, length and width of egg, and egg float—from 15 randomly selected individuals were analyzed. Eggs were placed on a wet filter paper and measured under the microscope with an ocular micrometer (Unilab GE-34, Binocular Research Microscope, India) and recorded. The egg shape, ridge numbers and floats were studied following earlier protocols [30, 31]. In isofemale lines, egg ridges were counted in every alternate generation.

Measurement of wing length

A total of 15 wing samples of both male and female mosquitoes were selected for wing measurement. The wings of male and female mosquitoes were carefully dissected 10 days after eclosion. The mosquitoes were anesthetized on CO_2 pads and the right wing dissected under a microscope (Olympus, SZX2-ILLK, Germany). Additional care was taken to ensure the wings were not damaged or folded during mounting. The wings were measured from the distal end of the alula to the tip between R1 and R2 veins, excluding the fringe scale as they are normally curved or wither off during mounting [32]. Wings were photographed and recorded under a stereomicroscope (Leica MZ10F, Germany). The photographed wings were measured using Leica Application Suite X (LAS X) software. The measurements were carried out independently by two observers to minimize error.

Estimation of homozygosity

The Fastq files for the three reference genomes were downloaded from NCBI SRA (sequence read archive). The three genomes (IndCh = SRR15146350, IndInt = SRR15603373, STE2 = SRR1168951) were mapped against the UCI 2.0 reference genome using Bowtie2 to call variants and generate a VCF file. These files were filtered for DP = 3, QUAL = 10 and Chr 2, 3, X and MAF (minimum allele frequency) = 0.01 and merged. The merged VCF file was converted to a matrix file containing numeric genotype information (0, 1, 2) representing the three possible genotypes for each SNP. The information was used to quantify heterozygosity-homozygosity in the genomes.

Insecticide-susceptibility assays

Five-day-old adult female mosquitoes of the parental and isofemale colonies were used for insecticide-susceptibility assays as per WHO guidelines. Insecticide-impregnated papers of discriminating doses and insecticide resistance monitoring test kits were ordered from Vector Control Research, Universiti Sains Malaysia (a WHO referral center for these kits and impregnated papers) [33].

Each replicate consisted of 25 females, and four such replicates were used for the experiment. One corresponding control was tested with the control papers supplied with the kit for respective classes of insecticides. They were exposed to eight insecticides representing all four major insecticide classes (Table 1). Mosquitoes used for DDT, permethrin and deltamethrin were pre-exposed to PBO (synergist) to understand the role of the metabolic mechanism of resistance. Assays were performed with 1 h exposure followed by 24 h recovery time, where the mosquitoes were provided with 10% glucose solution soaked on cotton pads. The mortality was recorded 24 h post-exposure, and all the mosquitoes (dead and alive tested) were kept in individual microfuge tubes and preserved at − 20 °C for future analysis. All the susceptibility assays were carried out at the TIGS insectary at 27 ± 1 °C and 75 ± 5% RH.

Vectorial competence studies

The vector competence studies were carried out in parental and isofemale colonies. The competence was

Table 1 List of insecticides, their respective classes and concentrations used for the insecticide-susceptibility assays

Sl. no.	Insecticide	Class	Discriminating concentration (w/v)
1	DDT	Organochlorines	4%
2	Dieldrin		4%
3	Malathion	Organophosphates	5%
4	Fenitrothion		1%
5	Bendiocarb	Carbamates	0.1%
6	Propoxur		0.1%
7	Permethrin	Synthetic pyrethroids	0.75%
8	Deltamethrin		0.05%
9	PBO	(Synergist)	4%

evaluated in both in vivo and in vitro assays using the *P. berghei* and *P. falciparum* parasites.

Plasmodium berghei

Albino mice (BALB/c) were provided by the Animal Care Resource Center (ACRC) of the National Centre for Biological Sciences (NCBS), Bangalore, India. All animal procedures were approved and conducted according to the Instem IAEC under project INS-IAE-2019/01(N). The rodent malarial parasite *P. berghei* (ANKA strain-MRA-311, BEI Resources, USA) was used to infect mice by injection of 100–150 µl intraperitoneally per mouse [34]. Infected mice, upon reaching 5–6% parasitemia and 1:1 or 1:2 male:female gametocyte ratio, were anesthetized and exposed to 5–6-day-old female mosquitoes as detailed below. Prior to keeping mice for feeding mosquitoes, the final counts of parasitemia and exflagellation were recorded [34].

After eclosion, adult female mosquitoes were provided with 10% glucose solution for 4–6 days. Forty mosquitoes in two replicate cups were kept fasting for 6 h prior to blood feeding. The mosquito cups were covered with a black cloth and left undisturbed for 30–40 min during blood feeding. The fully engorged females were separated from unfed and half-fed mosquitoes. The cups were maintained in a bioenvironmental chamber (Percival insect chamber I-30VL, Perry, IA, USA) at 19 °C and 75% RH (12:12 h day and night cycles) for 13–14 days. A cotton wool pad was soaked with 10% D-glucose; 0.05% para-aminobenzoic acid (PABA) solution was changed every alternate day until dissection. The mosquito midguts were dissected on the 14th day using PBS and stained with 1% mercurochrome. Midguts were examined for the presence of oocysts and recorded under a microscope (Nikon eclipse Ni-U upright microscope, Japan).

Plasmodium falciparum

Plasmodium falciparum [PfNF54 Line E, procured from BEI resources (MRA-1000)] was revived for standard membrane feeding assay (SMFA). The culture was initiated at 0.15–0.2% asexual parasitemia and 5% hematocrit in 10 ml complete medium (RPMI-1640 with 6 g/l HEPES, 50 mg/l hypoxanthine, 2.5 g/l sodium bicarbonate and 10% human serum) (ethics approval ref. no. inStem/IEC-12/001, dated 20/03/2019). The cultures were maintained in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for 16–18 days with daily media change. For the mosquito feeding experiment, staggered cultures (14 and 17 days old) were selected based on their stage-V gametocytemia and exflagellation activities (above 10 ex-flagellating centers per 40× field) and pooled [35].

During the day of SMFA, 4–6-day-old female mosquitoes (40 nos., in duplicate) were placed in paper cups and maintained with 5% D-glucose overnight. Sugar cotton was removed 4 h prior to blood feeding, and mosquitoes were kept in the dark till SMFA [36]. They were fed with mature stage-V *P. falciparum* NF54 gametocytes as mentioned earlier. The temperatures were maintained at 37 °C during SMFA. After 30 min of blood-feeding, fully engorged mosquitoes were maintained in an environmental chamber set at 26 °C and 75% RH (Percival I-30VL insect chamber, Perry, IA, USA). Mosquitoes were provided fresh wet cotton balls soaked in 10% D-glucose and 0.05% PABA solution daily. The midguts were dissected on the 9th day and stained with 1% mercurochrome, and the number of oocysts per midgut was recorded using a light microscope (Nikon Eclipse Ni-U upright microscope, Japan).

Statistical analysis

Descriptive and inferential statistical analyses were used in the present study. Various life table parameters such as fecundity, hatchability, pupation percentage, eclosion percentage and male:female ratio were recorded and are presented in graphical forms. For eggs and wing measurements, statistical t-test analysis for independent or correlated samples was performed using Vassar Stat software (http://vassarstats.net/). The mean and SEM were compared between populations. A p-value > 0.05 was considered non-significant for each parameter. Insecticide susceptibility was evaluated using the Prism GraphPad software package. The vectorial competence between the mosquito populations was analyzed using a non-parametric Mann-Whitney test using Prism GraphPad.

Results

Transgenerational fitness during sibling mating

In each generation, several key life table parameters such as fecundity, percent egg hatchability, number of larvae and pupae, sex ratio (male:female), longevity of adult females (lifespan) and frequency of blood meal were recorded. A greater number of females was selected to start the isofemale line as we expected higher mortality during the initial filial generations due to inbreeding [37, 38]. The average number of eggs was around 80–100 per individual female, and fecundity of both the lines normalized around the 15th generation (Fig. 2A). A similar trend was also observed in hatching percentage (Fig. 2B). Post normalization, the hatchability of both isofemale lines improved to 85%. The development of larvae to pupae (pupation %) (Fig. 2C), followed by the transformation of the pupae to adults (eclosion %) (Fig. 2D), was affected during sibling mating. There were instances where sharp dips were observed owing to the mortality of the larvae and pupae. Most of the pupal mortality was observed during eclosion as also observed in other studies [39].

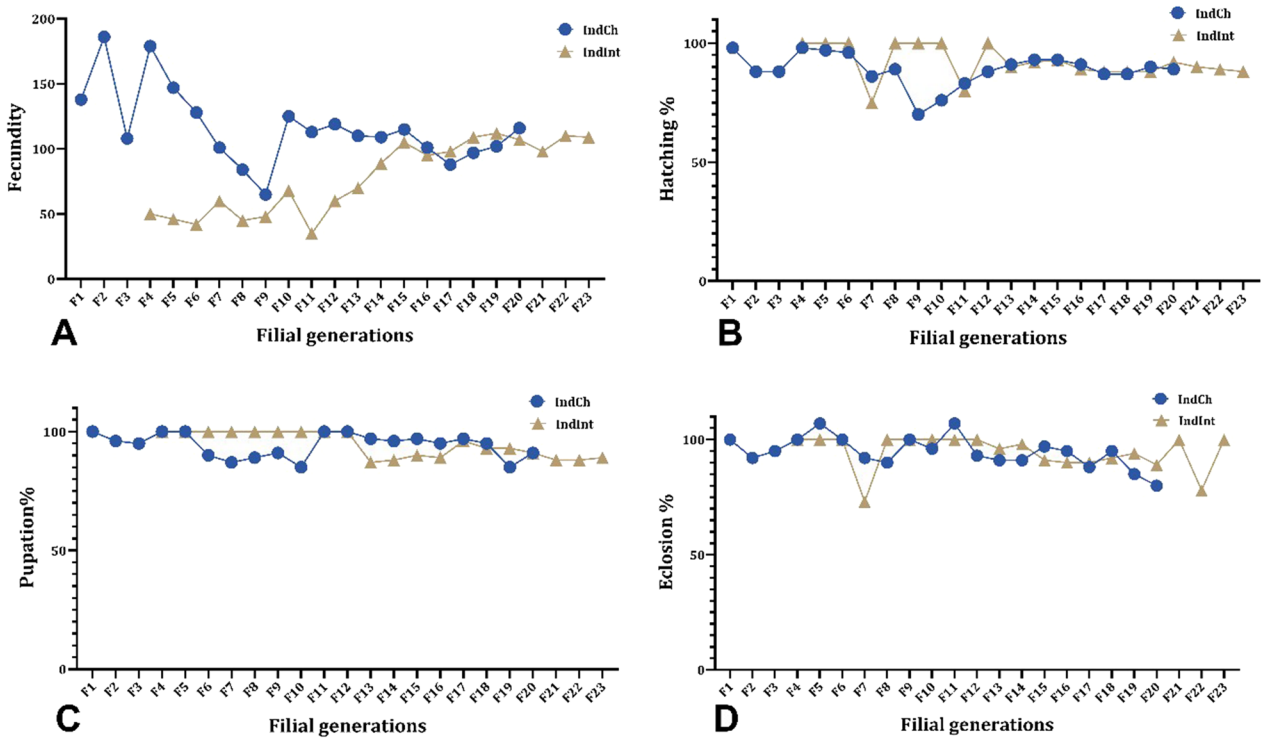

Fig. 2 Graph of the two isofemale lines of *An. stephensi* (IndCh: F1–F20 generations and IndInt: F4–F23 generations) showing **A** transgenerational fecundity, **B** hatching %, **C** pupation % and **D** eclosion % across filial generations

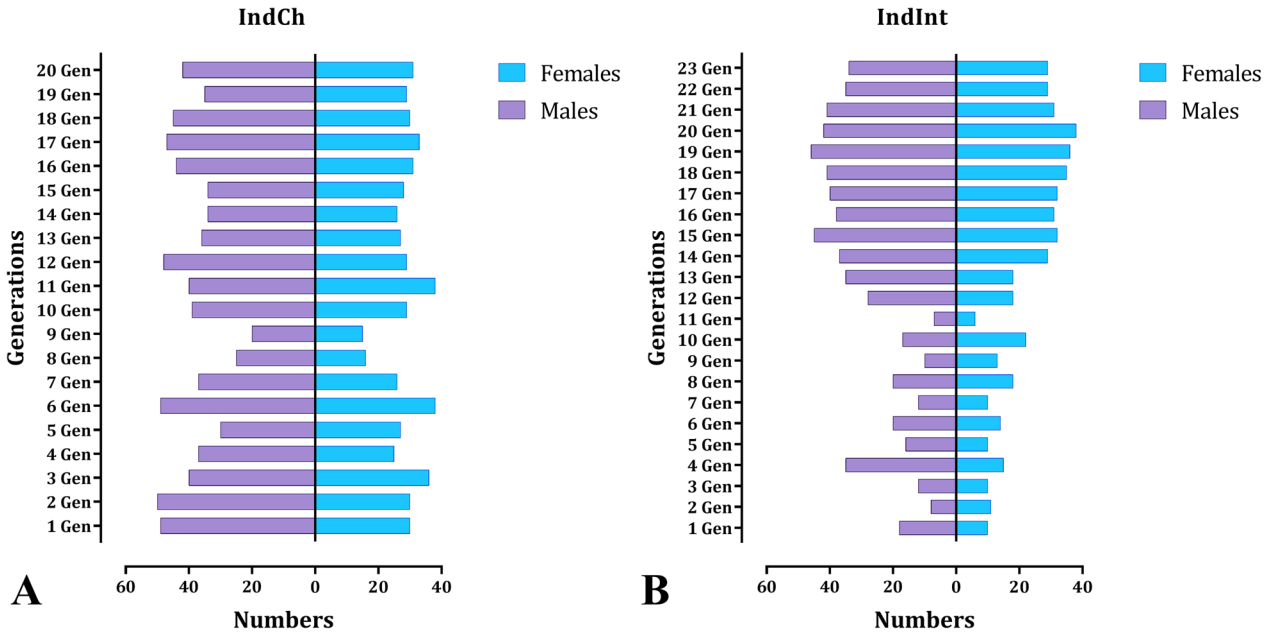

Fig. 3 The male:female numbers among the two isofemale lines **A** IndCh isofemale and **B** IndInt isofemale across filial generations (up to 20 and 23 generations, respectively)

Table 2 Egg measurement of paternal and isofemale strains of IndCh and IndInt biological forms

Parameter	T2	IndCh	T6	IndInt
EL	442.38 ± 3.3	443.53 ± 4.84	417.05 ± 4.51	418.66 ± 3.36
EW	134.7 ± 2.12	150 ± 2.92	128.82 ± 1.89	130 ± 2.18
EL/EW	2.96 ± 0.06	3.30 ± 0.06	3.21 ± 0.04	3.22 ± 0.05
EFL	218.09 ± 3.49	229.41 ± 4.81	210.58 ± 6.21	209.33 ± 10.62
EFW	64.76 ± 1.48	71.17 ± 6.96	67.64 ± 7.52	69.33 ± 7.98
EFL/EFW	3.40 ± 0.09	3.24 ± 0.08	3.14 ± 0.12	3.15 ± 0.15
FR	20.8 ± 0.11	20.81 ± 0.08	17.35 ± 0.31	16.66 ± 0.13
EL/EFL	2.04 ± 0.03	2.0 ± 0.07	2.13 ± 0.11	1.97 ± 0.06
EFL/ER	10.49 ± 0.19	10.98 ± 0.22	12.20 ± 0.43	13.02 ± 0.52
EFL/EW	1.46 ± 0.03	1.7 ± 0.03	1.64 ± 0.05	1.61 ± 0.06
EFW/ER	3.11 ± 0.07	3.41 ± 0.08	3.92 ± 0.13	4.16 ± 0.12
EW/EFW	2.34 ± 0.07	1.90 ± 0.04	1.92 ± 0.05	1.93 ± 0.08

EL (length of egg), EW (width of egg), EL/EW (length/width of egg), EFL (egg float length), EFW (egg float width), EFL/EFW (egg float length/egg float width), FR (float ridge no.), EL/EFL (egg length /egg float length), EFL/ER (egg float length/ egg ridge), EFL/EW (egg float length/egg width), EFW/ER (egg float width/ egg ridge), EW/EFW (egg width/egg float width)

The male:female ratio was calculated in each generation. The findings suggest (Fig. 3A, B) that the ratios tend to get skewed during the first few filial generations and balance out after successive generations (1:1.5) [23]. There was no significant change in the longevity of adult females (lifespan) or in their blood-feeding behavior.

Morphometrics of eggs and wings

To ascertain the morphometric changes that arise during and after sibling mating, several parameters such as egg shape, size, float numbers, float ridge and wing length were observed and recorded (Table 2). The egg was slightly boat-shaped, black in color and blunt at the anterior and posterior ends (Fig. 4). The floats, which are an important morphological marker for differentiating the biological forms of different *Anopheles* species, showed minor variations in size. The maximum float lengths of IndCh and IndInt isofemale forms were 229.41 ± 4.81 and 209.33 ± 10.62 µm and the float widths 71.17 ± 6.96 and 69.33 ± 7.98 µm, respectively. The float length-to-width ratios of IndCh and IndInt were 3.24 ± 0.08 and 3.15 ± 0.15, respectively. The maximum float lengths of IndCh and IndInt parental strains were 218.09 ± 3.49 and 210.58 ± 6.21 µm and the float widths 71.17 ± 6.96 and 69.33 ± 7.98 µm, respectively. The float length-to-width ratios of IndCh and IndInt parental strains were 3.40 ± 0.09 and 3.14 ± 0.12, respectively (Table 2). The float size also influenced the number of ridges. For type and intermediate isofemale eggs, the number of float ridges was 20.81 ± 0.08 (range 20–21) and 16.66 ± 0.13 (range 16–17), respectively. For type and intermediate parental strains, it was 20.8 ± 0.11 (range 19–21) and 17.35 ± 0.31 (range 16–18). Many other parameters related to morphometry of eggs are presented in Table 2. Overall, there were no significant differences in all the parameters of eggs when compared between the isofemale lines of IndCh ($F = 1.03$, $p > 0.05$) and IndInt ($F = 1.01$, $p > 0.05$) and also when compared with their respective parental lines.

There was no significant difference between the male and female mosquito wing lengths (Table 3) and between the parental line and their isofemale forms. The wing measurement values were non-significant when compared between the isofemale lines of IndCh ($F = 1.32$, $p > 0.05$ for males; $F = 2.43$, $p > 0.05$ for females) and IndInt ($F = 1.31$, $p > 0.05$ for males; $F = 1.12$, $p > 0.05$ for females) and with their respective parental lines.

Homozygosity achieved in isofemale lines

To decipher the levels of homozygosity achieved through sibling mating across filial generations, we obtained 100× sequence coverage using Illumina reads from DNA pooled from 50 individuals from the isofemale IndCh and IndInt strains homogenized for 5 and 14 generations, respectively. As a negative control, we used the

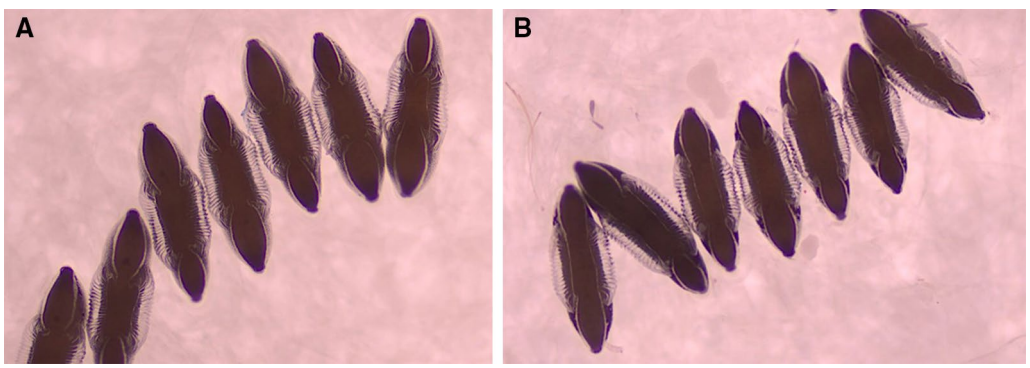

Fig. 4 Eggs of *An. stephensi* (magnification 10×). **A**. IndCh **B**. IndInt

Table 3 Wing measurement of paternal and isofemale strains of IndCh and IndInt variants

Mosquito strains	Male				Female			
	L		W		L		W	
	M ± SEM	SD	M ± SEM	SD	M ± SEM	SD	M ± SEM	SD
T2	3.07 ± 0.03	0.0832	0.58 ± 0.03	0.0401	3.28 ± 0.03	0.1144	0.76 ± 0.01	0.0178
IndCh	3.22 ± 0.03	0.1354	0.71 ± 0.02	0.0931	3.43 ± 0.05	0.1962	0.81 ± 0.03	0.1239
T6	2.91 ± 0.01	0.1781	0.56 ± 0.02	0.0737	3.23 ± 0.05	0.2194	0.746 ± 0.02	0.0865
IndInt	3.16 ± 0.03	0.1049	0.67 ± 0.02	0.0679	3.35 ± 0.05	0.2151	0.78 ± 0.01	0.0494

dataset generated from a pool of 50 + individuals directly from a laboratory colony [40] of the STE2 strain without genetic homogenization. The low ratios of heterozygous over homozygous SNPs seen for the two isofemale lines showed the decrease in heterozygosity achieved by isofemale lines around the fifth generation over pools of individuals from laboratory samples (Fig. 5).

It should be mentioned that while IndCh is homozygous for standard forms of all known inversions, STE2 is heterozygous for the 2R*b* genotype and IndInt is heterozygous for the 3L*i* genotype even after 14 generations of isofemale selection [44]. Interestingly, the isofemale line for IndInt shows increased heterozygosity for 3L*i* relative to its parent line (unpublished data), perhaps amplifying the reduction in vectorial competence in the isofemale over its parental line (T6, Figs. 7, 8). Even in the case of IndCh, the percentage of insects with the standard 2R*b* increased over generations of isofemale lines [33], perhaps explaining the increased vectorial competence of the isofemale line over that of the parental line (T2, Figs. 7, 8).

Susceptibility assay across four classes of insecticides

Insecticide susceptibility was determined for both isofemales and their parental colonies across four classes of insecticides [33] (Fig. 6). The number of surviving individuals at 24 h post-exposure was calculated to understand the susceptibility/resistance status. The results suggest both the isofemale lines showed higher susceptibility to DDT than their parental lines. In case of dieldrin, IndCh showed a decreased mortality percentage of 71.6% compared to T2, which exhibits possible resistance. In contrast, the IndInt showed greater

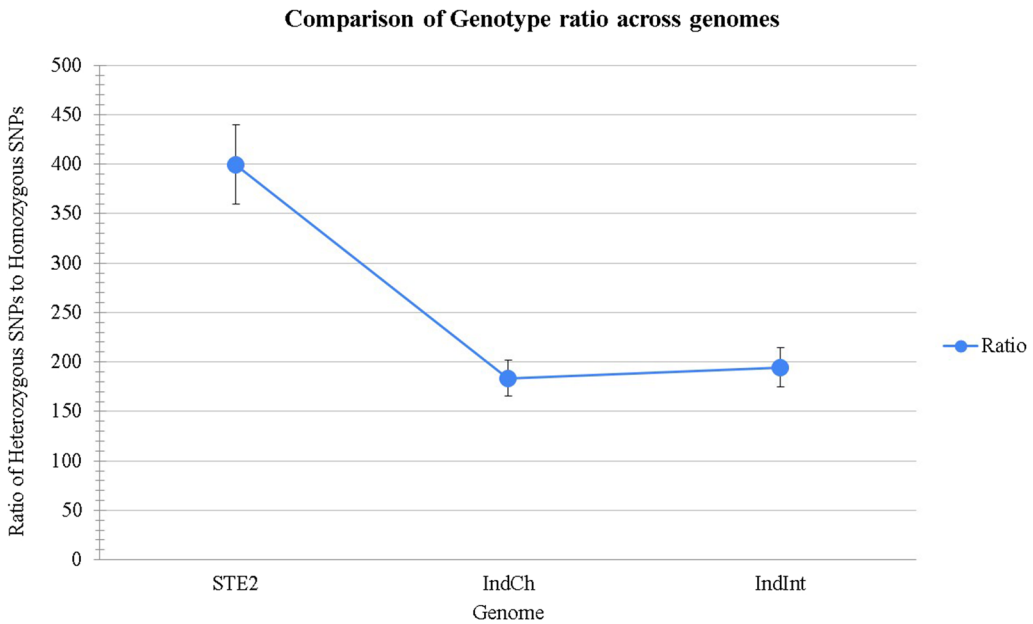

Fig. 5 Ratio of heterozygous versus homozygous SNPs in the three strains including STE2 (not homogenized), IndCh (homogenized over 5 generations) and IndInt (homogenized over 14 generations)

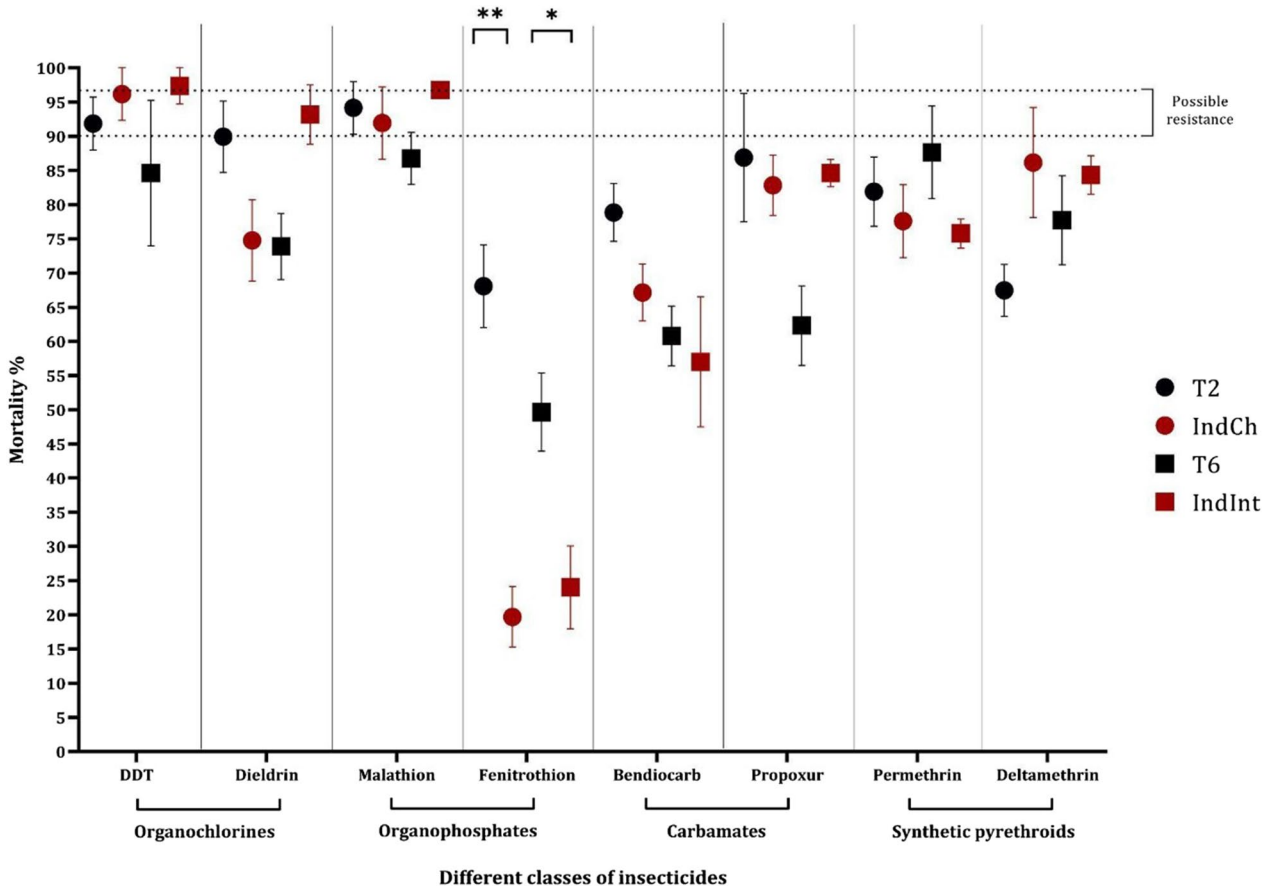

Fig. 6 Summary plot of mortality percentage across four different classes of insecticides

sensitivity (93% mortality) than T6 (68.2% mortality) as per WHO standards [60]. The change in the susceptibility might be due to different target sites of these two insecticides. In organophosphates, all the populations showed high mortality (> 90%), except T6. For fenitrothion, both isofemale lines exhibited significant resistance (< 30% mortality) compared to their respective parental lines (non-parametric, Mann-Whitney test; $p < 0.0001$). For carbamate and synthetic pyrethroids, all four mosquito colonies showed confirmed resistance (mortality < 90%, range 60–87%) (Fig. 6).

Vectorial competence studies

Plasmodium berghei

Four colonies (T2, IndCh, T6, IndInt) were fed with *P. berghei*-infected blood of BALB/c mice and evaluated for their infection rate. The infection rate was calculated for each colony as the percentage of mosquitoes with oocysts in their midguts. There was not much variation in mean infection rates of the type form T2 (82.73%) and IndCh (85.07%) (Fig. 7). However, it was interesting to note there were considerable differences in the mean infection rates between the intermediate form of T6 (92.86%) and IndInt (70.83%).

Among the four colonies, the highest oocyst range was observed in T2 (1-1050), followed by IndCh (1-1003), T6 (1-870) and IndInt (1-150) (Fig. 7B). The mean number of oocysts per positive midgut in T2, IndCh, T6 and IndInt was 52.47 (SD ± SEM = 120.3 ± 10.20), 235.6 (266.4 ± 32.55), 112.4 (168.6 ± 17.03) and 14.54 (24.39 ± 2.87), respectively, whereas the median values were 10.00, 190.00, 50.50, 5.00 for T2, IndCh, T6 and IndInt, respectively. Furthermore, a comparison between parental colonies and isofemale colonies revealed a significant difference in their vector competence ($p < 0.0001$, non-parametric, Mann-Whitney test).

Plasmodium falciparum

In the case of *P. falciparum*, there was little variation in mean infection rates of the type forms, T2 (80.40%) and IndCh (78.57%) (Fig. 8B). The mean infection rates differed between the intermediate form of T6 (79.62%) and

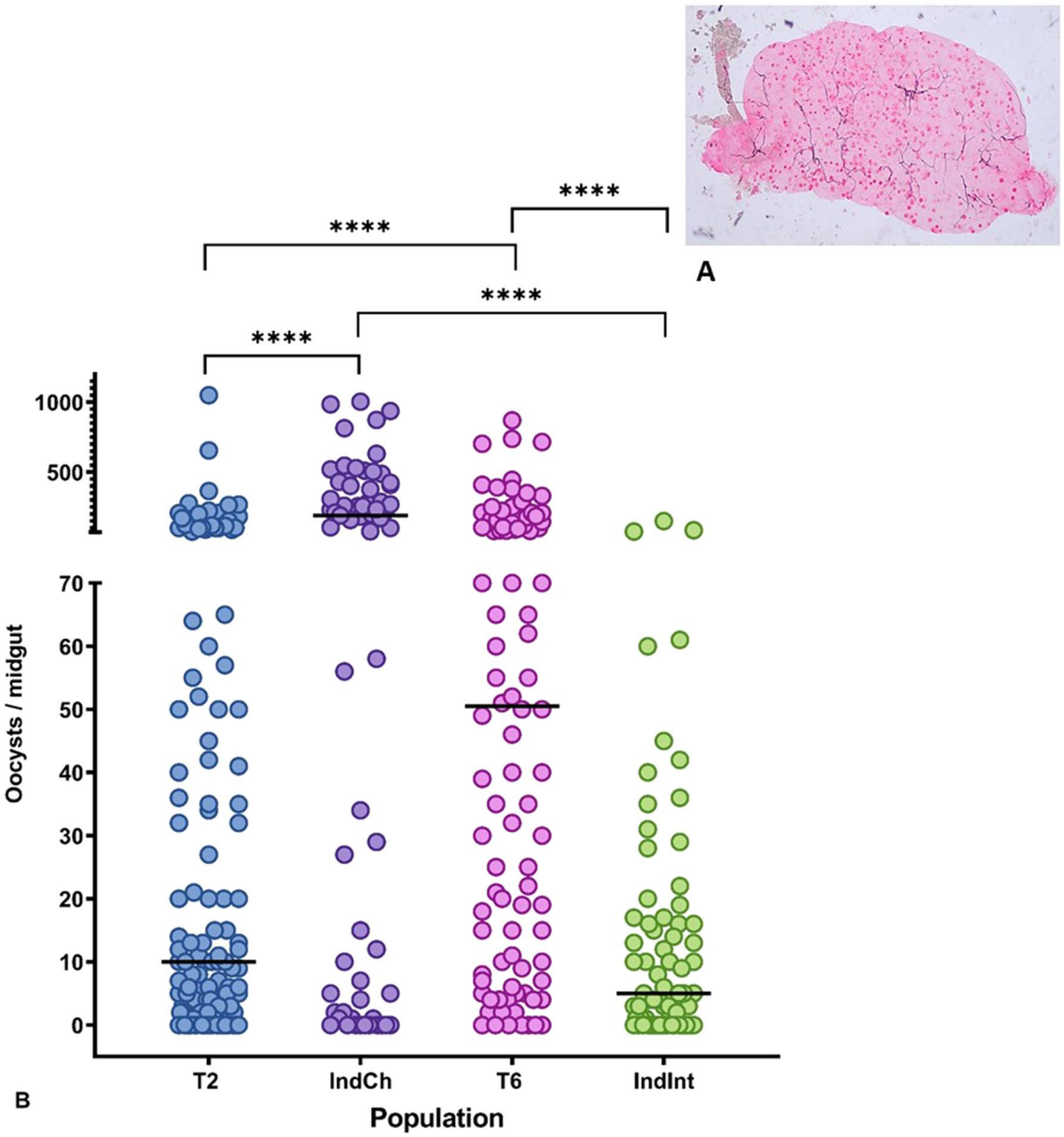

Fig. 7 **A** Representative image of *Plasmodium berghei*-infected mosquito midgut. **B** Vectorial competence between the parental and isofemale lines for *P. berghei*. Each dot represents the number of oocysts in an individual midgut of mosquitoes, with median values (horizontal bar). Asterisks denote significant differences at $p < 0.0001$ (Mann-Whitney non-parametric t-test)

IndInt (69.57%). Among the four colonies, the highest oocyst range was observed in T6 (1-231), followed by IndInt (1-125), IndCh (1-106) and T2 (1-85). The mean number of oocysts per positive midgut in T2, IndCh, T6 and IndInt was 16.34 (SD ± SEM = 17.94 ± 1.13), 26.39 (34.70 ± 2.77), 18.39 (19.56 ± 1.65) and 13.30 (19.63 ± 1.67), respectively, whereas the median values were 12, 14, 16, 6 for T2, IndCh, T6 and IndInt, respectively.

Furthermore, a non-parametric Mann-Whitney comparative test between T2-T6 colonies ($p < 0.0563$) and T2-IndCh colonies ($p < 0.3968$) did not show any significant difference. However, the homozygous forms of the isofemale colonies, IndCh and IndInt showed a

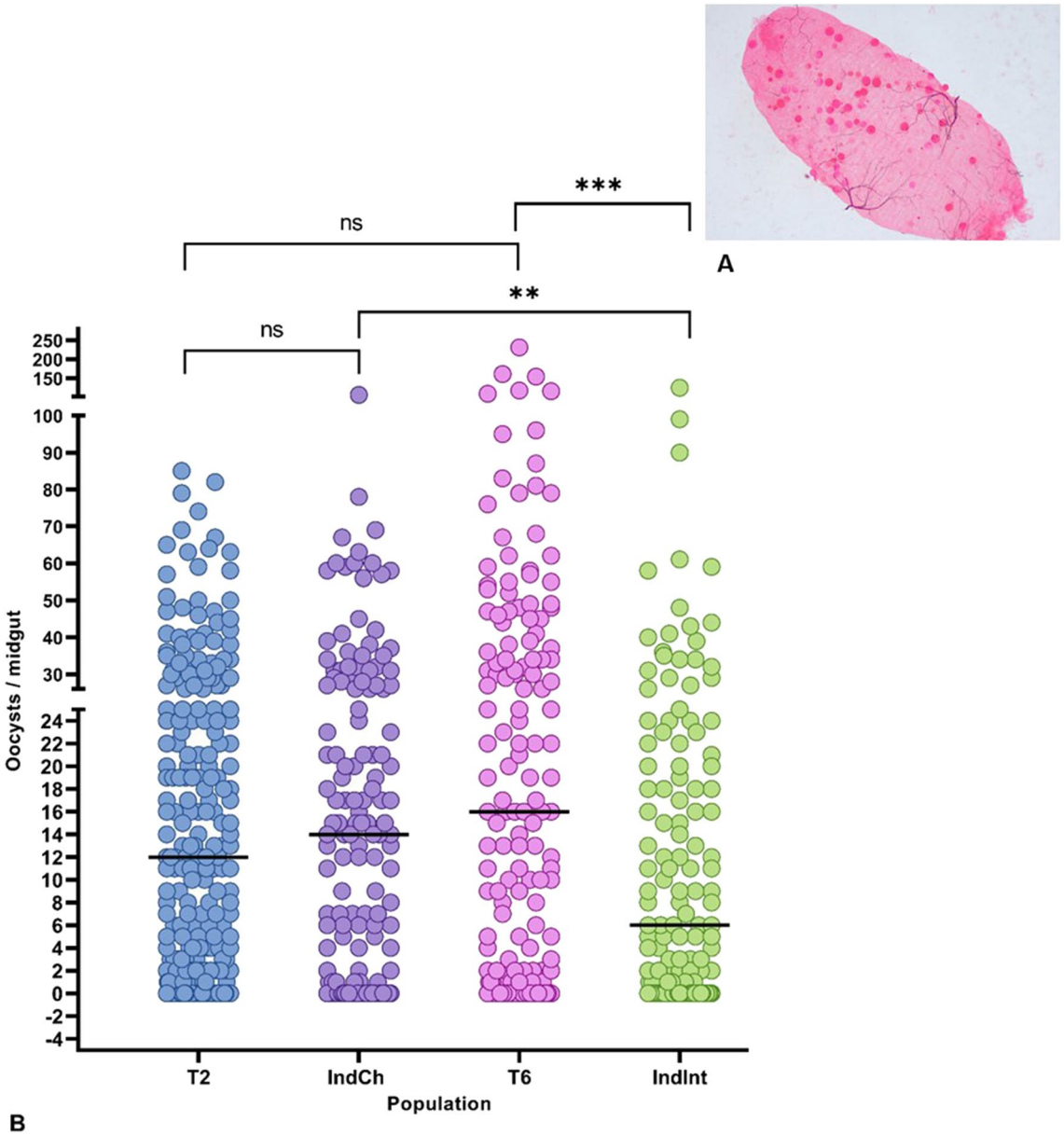

Fig. 8 **A** Representative image of *Plasmodium falciparum*-infected mosquito midgut. **B** Vectorial competence between the parental and isofemale lines for *P. falciparum*. Each dot represents the number of oocysts in an individual midgut of mosquitoes, with median values (horizontal bar). Asterisks denote significant differences at $p < 0.0001$ (Mann-Whitney non-parametric t-test)

significant difference ($p < 0.0045$). Significant difference was also observed between T6 and IndInt ($p < 0.0003$). These results corroborate earlier findings that the type form is a better vector than the intermediate form [34, 41] (Fig. 8B).

Discussion

The present study provides a detailed overview of the key considerations followed during the establishment of the isofemale line in the two biological forms of *An. stephensi*. We have meticulously characterized several life cycle parameters in every generation during the creation of both isofemale lines to maintain their authenticity. During establishment, key emphasis was given to restore some characteristics of the parent population after the initial bottleneck to achieve normalization of fecundity, hatching, pupation, eclosion and male:female ratio. Accordingly, we found that in both isofemale lines, homogeneity in these parameters was achieved within ~ 13 generations (Fig. 2).

Even though homogeneity in the biological parameters was achieved in ~ 13 generations, the impact of this on other traits, such as insecticide resistance and vectorial competence, was dramatic. This is the first time to our knowledge that a discrete study has been conducted by establishing two homozygous isofemale lines from the type and intermediate forms of *An. stephensi* and assessing their vectorial competence against two different malaria parasites, *P. berghei* and *P. falciparum*. The findings suggest that there is significant enrichment in vectorial competence between the parental and corresponding isofemale lines. When mosquitoes were exposed to *P. berghei*-infected mice, there was increased susceptibility to the type (IndCh) compared with intermediate form (IndInt). Furthermore, a significant difference in vectorial competence was seen between the parental and corresponding isofemale lines. This might be due to the high levels of homozygosity in certain alleles enhancing the vectorial competence.

For the *P. falciparum*-infected blood meal, there was no significant difference between the parental and corresponding isofemale lines. However, significant differences were found between the two isofemale lines belonging to the type and intermediate forms, supporting earlier reports of the type form being a better vector than the intermediate form [25]. Establishing an isofemale line has been shown to delineate the non-additive genetic variance due to local adaptations, bottleneck events and other epistatic effects [42]. We conclude that isofemale lines derived from type and intermediate forms achieve enrichment of characteristic phenotypes regarding vectorial competence expected for each form.

We also sequenced DNA from both IndCh and IndInt lines after 5 and 14 generations to assess the homogeneity achieved at the genome level. As a negative control, we used a public dataset with 100× coverage of STE2 strain. The ratio of the heterozygous versus homozygous variants in these reads relative to a gold-standard reference genome [43] clearly shows that genome level homozygosity is achieved within as few as five generations in IndCh. Furthermore, the increased homozygosity in the organism provides an opportunity to decipher the genome architecture at very high resolution [43, 44]. While the high-resolution genome assemblies of the UCI and IndCh strains were achieved after 5 and 6 generations of isofemale lines, the IndInt line was homogenized over 14 generations before attempting an assembly because, as shown in Fig. 2A, increased generations were needed to normalize fecundity. This could be because, unlike the UCI and IndCh strains that have no heterozygous paracentric inversions, IndInt retains a large paracentric inversion even after 14 generations. Furthermore, our data show (unpublished data) that the percentage of this inversion increased considerably with more generations relative to that of the parent population. This may be a notable example of the complexity of maintaining a given trait while retaining fitness.

As a comparative account between *P. berghei* and *P. falciparum*, we also observed higher infection rates in both the parental and isofemale lines for *P. berghei* model. However, we did not find a significant difference between the two parental lines T2 and T6 and also the parental line T2 and its isofemale form for *P. falciparum* infections. We speculate that there might be differential immune response that could have happened during homogenization, leading to the possibility of some anti-*Plasmodial* genes being differentially expressed. In our earlier work, we have described few structural variations (2Rb and 3Li inversions) and genes associated with vector competence during the genomic characterization of these isofemale lines, IndCh and IndInt [44, 59]. Since functional genomics was not in the scope of this study, we did not explore it further. However, probing this in greater detail would shed some light on delineating the specific host-parasite interactions.

In the present study, we observed significant variations in insecticide resistance between the founding population and its corresponding isofemale progeny. This might be a result of the segregation of the genotypes resulting in the better demarcation of the susceptible/resistant phenotype compared with the parental lines. This can be further studied for a better understanding of underlying mechanisms of resistance, which is relatively easier to identify in isofemale lines because of their homozygosity.

In all four colonies including the two parent and two isofemale lines, the levels of resistance varied across the two insecticides belonging to the same class, such as DDT and Dieldrin, belonging to the organochlorine class. For Dieldrin, the parental line T2 exhibited greater sensitivity than its isofemale IndCh, whereas the parental line T6 showed greater resistance than its isofemale form IndInt. The differences in the modes of action of these two insecticides might be the reason for this trend. For example, DDT resistance is associated with mutation(s) in the target site of the voltage-gated sodium channel (vgsc) gene and metabolic resistance [45, 46], whereas dieldrin resistance is due to a single point mutation in GABA receptors [47]. Also, among the organophosphates, resistance was observed to fenitrothion, but susceptibility was seen for malathion among all four colonies. For fenitrothion, there was a significant difference in the susceptibility status between the parental and their respective isofemale lines. Earlier studies had suggested that many strains of *An. stephensi* collected from different parts of India were susceptible to fenitrothion [29]. However, for carbamates and synthetic pyrethroids,

all four populations showed resistance to both classes of the insecticides. It is interesting to note that although the insectary-colonized mosquitoes and their isofemale lines were not under any selection pressure from insecticides and were grown without any insecticide exposure, they continued to maintain this resistance.

The observed variations in insecticide susceptibility might be due to large structural variants such as inversions. Recent advances have revealed the possible role of structural variations like duplications and inversions in insecticide resistance, which would be interesting to dissect among isofemale colonies in the future [48]. The role of structural variation has also been implicated in different mechanisms of copy number variation, transposable element insertions and tandem duplication of different metabolic genes [49, 50]. Characterization of the isofemale lines during their establishment as reference genomes revealed that there is a homozygous standard 2R*b* form in the IndCh isofemale line [44] and a heterozygous 3L*i* inversion in the IndInt isofemale line [59]. The functional characterization of the 3L*i* region revealed 36 isoforms of cuticle-forming genes in the IndInt isofemale line. Earlier studies suggest that cuticle thickening is associated with pyrethroid and insecticide resistance in other *Anopheles* species [51]. Similarly, the 2R*b* region has 1353 predicted genes, which include several genes associated with insecticide resistance such as ACE1 as well as tandem clusters of GST and Cyp450 paralogs in the IndCh isofemale line [44].

Variations in vector competence among the biological forms

Although the various biological forms can be clearly delineated using the number of ridges on the egg float, there have been efforts to develop molecular markers to identify the various forms. These include mitochondrial genes cytochrome oxidase 1 (COI) and cytochrome oxidase 2 (COII) [52], the rDNA-ITS2 and domain-3 (D3) marker [53], odorant-binding protein *Anste*Obp1 marker [54] and microsatellite markers [55] to characterize the different biological forms. A single SNP has been previously reported [54] to distinguish between the various forms of *An. stephensi* found in Iran. When we tested the validity of this SNP for Indian *An. stephensi* populations (insectary-colonized and wild populations) that were collected from five different locations, we found that most of the individuals from the wild had the SNP (G) in *AsteObp1* (intron I, position 91 bp) reported to be diagnostic of the type form, 18/105 individuals had an A at this same position, 18/105 had an A/G and 1/105 had an A/C in that position, likely representing population-level variations that have not been reported earlier (unpublished data). Also, many of the other markers are not universal and may vary from region to region and cannot resolve the biological forms accurately [56]. Here, we clearly demonstrate that the number of egg ridges remains the gold standard and applicable marker to develop isofemale lines until new molecular markers are identified.

There are also reports of a third form with the lowest egg-float ridge number known as 'var. *mysorensis*' [25, 31, 34, 57], but this has not been established or characterized in the current study. The *mysorensis* form is mostly found in rural areas and breeds in fresh water sources like wells, ponds, cement tanks, streams, etc. Earlier studies have shown var. *mysorensis* to exhibit variations in their insecticide susceptibility status [58] and vectorial competence [41]. The *mysorensis* form is highly zoophilic and has very poor vectorial competence. A thorough characterization of this third biological form might reveal cues that can be exploited in vector management strategies.

Conclusion

Overall, the findings of this article show the benefit of developing isofemale lines from the insectary-colonized mosquito populations. Furthermore, we showed that as few as five filial generations of sibling mating may be sufficient in *An. stephensi* to achieve greater levels of homozygosity. However, more sibling matings may be required (> 13 generations) to establish isofemale lines with optimal fitness. The isofemale lines thus developed could be used to elucidate mechanisms underlying insecticide resistance, which is more pronounced than in the parental lines. The isofemale lines should also prove useful in understanding the basis for the greater vectorial competence of the type variant relative to the intermediate form for both *P. berghei* and *P. falciparum*. By assembling high-quality reference genomes for IndInt and IndCh, we have been able to hypothesize the genetic factors/structural variants that might account for the differential vectorial competence [59].

Acknowledgements

The authors thank Dr. Rakesh Mishra, Director of the Tata Institute for Genetics and Society (TIGS), India, for his support. We are thankful to Dr. Susanta Kumar Ghosh, Senior Scientist (retired) of NIMR-ICMR, Bangalore, for guiding the establishment of TIGS insectary. We are grateful to Dr. Kailash Patra, R&D Director at Intrivo Diagnostics, California, USA, for giving training on parasite cultures and Mr. Joydeep Roy for his technical support. Animal work in the NCBS/inStem Animal Care and Resource Center was partially supported by the National Mouse Research Resource (NaMoR) grant #BT/PR5981/MED/31/181/2012;2013-2016 and 102/IFD/SAN/5003/2017-2018 from the Department of Biotechnology, GoI. The authors thank Tata Institute for Genetics and Society (TIGS) India for funding researchers involved in this work and IBAB for bioinformatics work.

Author contributions
CG: Established isofemale lines, recorded life-table data, egg morphometric study and *P. berghei* vectorial competence assay. NK: *P. falciparum* vectorial competence assays, analyzed the data and insectary-related work. RBSK: Insecticide susceptibility assays. SM, SGJ, CKR: Insectary-related work and isofemale line maintenance. TA: Bioinformatic analysis. SSri: Overseeing bioinformatics work, helping in reviewing the manuscript draft. SS: Conceptualization, funding and contributions to the manuscript. SK: Conceptualizing and writing the first draft, analyzing the data and insectary-related work. SunS: Coordinating and overseeing the entire insect work and helping in reviewing the manuscript draft. All authors read and approved the final manuscript.

Funding
This research is funded by internal grants from Tata Institute for Genetics and Society in Bangalore, India.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
Ethics approval ref. no. inStem/IEC-12/001, dated 20/03/2019. Standard membrane feeding system (approval ref. no. TIGS 2nd IBSC Oct 2018).

Consent for publication
All authors have provided consent for publication.

Competing interests
The authors have no competing interests.

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Received: 16 November 2022 Accepted: 7 February 2023
Published online: 28 February 2023

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