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Organophosphate Insecticide Exposure Impacts Reproductive Success in Insensitive Acetylcholinesterase *Anopheles gambiae* Mosquitoes

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Extensive use of insecticides has led to the selection of resistance alleles in malaria vectors threatening the control programs. Even if mosquitoes are not killed directly in the contact of insecticide-treated bed nets, their capacity to transmit malaria parasite could be decreased because of the consequences on their life-history traits after repeated exposure. The current work investigated the effects of organochlorine, carbamate, organophosphate, and pyrethroid insecticide exposure on the reproductive success in *Anopheles gambiae s.s*. Two *Anopheles gambiae* strains, AcerKis, KisKdr, were used. According to WHO recommendations, female mosquitoes of these resistant strains were exposed to discriminant doses of DDT, chlorpyriphos-methyl, bendiocarb, and permethrin insecticides. Surviving mosquitoes were then fed and allowed to lay eggs. Fecundity was assessed by examining the number of eggs per mosquito, the number of larvae per egg batch and larval hatching rates were used to evaluate the fertility. The data showed that AcerKis females surviving chlorpyriphos-methyl exposure significantly laid few eggs. No significant difference in the hatching rate was noticed in AcerKis females exposed to bendiocarb compared to their control. No significant effect on the fecundity and fertility was observed in KisKdr females exposed to permethrin. Our finding showed that organophosphate insecticides represented here by chlorpyriphos-methyl could hamper egg-laying in insensitive acetylcholinesterase *An. gambiae* female mosquitoes. This knowledge could help design alternative vector control strategies targeting fecundity and fertility in resistant malaria vectors.

Keywords: insecticide exposure, resistant *Anopheles gambiae*, fecundity, fertility, malaria

INTRODUCTION

Malaria is still among the infectious diseases ranked in the top causes of death worldwide, especially in low-income countries (1). This parasitic disease is transmitted to humans by female mosquitoes of the genus *Anopheles*. Three strategies are developed for malaria control, including prevention, diagnosis, and treatment. To date, insecticide-based vector control through the use of Long-Lasting Insecticidal Nets (LLINs) and the Indoor Residual Spraying (IRS) is, among the prevention programs, the most widely implemented strategy and remains the most efficient method for malaria control in most endemic countries (2). Indeed, the percentage of the population protected by the vector control interventions increased from 29% in 2010 to 50% in 2018, representing half of the population worldwide (3).

The World Health Organization (WHO) recommended five classes of insecticides for vector control interventions, including pyrethroids, organochlorines, carbamates, organophosphates, and neonicotinoids (3). Unfortunately, the long-term success of both LLINS and IRS strategies is nowadays hampered by the widespread emergence of insecticide resistance mechanisms in mosquito populations, especially in sub-Saharan African regions (4–7). Besides, WHO reported that if there is no change in the current trend in malaria incidence (57 cases per 1000 population at risk), the predicted malaria case incidence would be 48 in 2025 and 42 in 2030, rather than 14 and 6 necessary to achieve the global technical strategy (GTS) pillars for malaria elimination by 2030 (3). Therefore, National Malaria Control Programmes (NMCPs), international policymakers, and funding agencies should make efforts to increase resistance monitoring and surveillance in Anopheline vectors (8). Since most of NMCPs developed in the endemic countries are in line with the malaria elimination and eradication goals, it appears essential that the implications of the insecticide resistance phenomenon in malaria transmission are much more explored and strategies to alleviate these effects are performed (4).

In most African countries, the major malaria vector *Anopheles gambiae* already displays a multi-resistance to the existing insecticides (9–11). However, in the absence of an effective malaria vaccine for individuals above 5 years old, there is still the need to look for alternative vector control strategies to protect the global population at risk.

Since the same insecticides recommended are still being used, the expanding coverage of vector control strategies leads to frequent contact of the resistant vectors with the insecticides (12). Therefore, these vectors can ingest higher doses of insecticides since the resistant alleles can allow them to prolong contact with treated materials (13) without being killed. Thus, one should ask whether the long-term accumulation of insecticide residues affects mosquito life-history traits. It was suggested that even if insecticides have no direct impact on the resistant vectors, they could significantly influence malaria transmission if they negatively affect the life-history trait of the vectors (14). Available data suggest that fitness costs are associated with insecticide resistance following insecticide exposure. This might influence malaria transmission by impacting mosquito life-history traits, such as reducing their lifespan (14). The discovery of much more life-history traits affected by the insecticide exposure would be useful for controlling resistant malaria vectors. Knowledge of vector biology has also been revealed to be important when implementing current control strategies. For instance, the understanding of mosquito blood-feeding and host-seeking abilities has allowed the development of vector control strategies such as i) zooprophylaxis to divert mosquitoes from human to nonhuman hosts set as bait (15); ii) Attractive Toxic Sugar Baits (ATSB), where mosquitoes are killed following feeding on toxic sugar meals to which they are attracted to (15); iii) Push-pull strategies that aim to reduce outdoor biting (16), by coupling the use of repellents with a baited trap to direct host-seeking mosquitoes from sprayed areas to the trap (17, 18).

However, little is known about the direct consequences of insecticide exposure on vector life-history traits, especially on the reproductive success of *Anopheles* mosquitoes. To contribute to the understanding of insecticide resistance implications for malaria vector control based on the mosquito reproductive system, this study investigated the effect of exposure to organochlorine (Dichlorodiphenyltrichloroethane), organophosphate (chlorpyriphos-methyl), carbamate (bendiocarb) and pyrethroid (permethrin) on the fecundity and fertility in two *Anopheles gambiae* strains.

MATERIALS AND METHODS

Mosquito Strains and Rearing

Two *Anopheles gambiae* sensu stricto (*An. gambiae* s.s.) laboratory strains already available at the insectarium of the Laboratory of Infectious Vector-Borne Diseases of the University of Abomey-Calavi in Ouidah (Benin) were used in this study. AcerKis strain is resistant to organophosphate and carbamate insecticides and is homozygous for the *ace-1*^{R} (G119S) allele (19). KisKdr, which is homozygous for the *kdr*^{R} (L1014F) allele, is resistant to pyrethroid and DDT insecticides (20). AcerKis and KisKdr were supposed to share the same genetic background but differ by the presence of resistance alleles (L1014F and G119S). All the colonies of *An. gambiae* were maintained at the insectarium under optimum conditions (27 ± 2°C temperature and 70 ± 8% relative humidity). Larvae were reared in the same conditions in dechlorinated water and were fed with Tetramin Baby fish food. After emergence, adult mosquitoes were fed *ad libitum* on a 10% honey solution and maintained in 30x30x30 cm cages until they were ready to be used for further assays.

Insecticide Exposure

Non-blood-fed mosquito females aged 3–5 days from all strains were exposed to discriminating dosages of several insecticides using the WHO standard vertical tube kits (21). Insecticide exposures were performed as follows: 1 hour exposure to bendiocarb 0.1% (BDC 0.1%); 30 minutes exposure to chlorpyrifos-methyl 0.4% (CM 0.4%); 3 hours exposure to permethrin 0.75% (PM 0.75%) and Dichlorodiphenyltrichloroethane 4% (DDT 4%). Control tests were also set up for each strain by exposing adult females to untreated papers. Test papers were used no more than five times before being replaced. After insecticide exposures, mosquitoes were gently

transferred into holding tubes, provided with a 10% honey solution and mortality was recorded 24 hours later.

Female Reproductive Success Assessment

After the holding period, AcerKis and KisKdr females were blood-fed on a rabbit and were allowed to blood feed again the third day after the first blood-feeding. Each blood-feeding session lasted for 30 minutes. The gravid mosquitoes of each strain were individually transferred into plastic cups containing wet Whatman filter paper for oviposition. They were allowed to feed on 10% honey solution until egg-laying. The number of females laying eggs was recorded, and eggs were counted under a stereomicroscope (Leica Microsystems EZ4HD). Egg batches (from individual females) were transferred in separate plastic trays filled with dechlorinated water, and the number of hatched larvae was recorded. Two biological replicates were performed.

Data Analysis

When control mortality is ≥ 5%, then the observed mortality in test groups was corrected using Abbott's formula, as follows (21):

$$\text{Corrected mortality} = \frac{(\%\ \text{observed mortality} - \%\ \text{control mortality})}{(100 - \%\ \text{control mortality})} \times 100$$

The WHO insecticide susceptibility test with discriminating concentration criteria was used to determine the phenotypic resistance status of the exposed mosquitoes to each class of insecticides (21). Insecticide resistance status was declared when the mortality rates were less than 90%. When the mortality rates were greater than 97%, mosquitoes were considered susceptible.

The fecundity in each mosquito strain was assessed as the total number of eggs over the total number of females that contributed to oviposition. Fertility in each mosquito strain expressed as a percentage of hatched larvae was evaluated by dividing the total number of first instar larvae over the total number of eggs. The median was used as the estimated parameters showed non-normal distribution (Shapiro-Wilk test: $p < 0.05$). Fecundity and fertility were compared with the Kruskal-Wallis test using R version 4.0.1 (www.cran.r-project.org). All the *p*-values were corrected for multiple comparisons by the Bonferroni method. The Chi-square test of independence of the package "Stats" was used to compare proportion where applicable and mortality rates using R software. All the analyses were set at a significance threshold of $p < 0.05$. Graphics were built using GraphPad Prism 8.0.2 software (San Diego, California USA).

RESULTS

Insecticide Susceptibility

A total of four hundred and eighty (480) *Anopheles gambiae* females of AcerKis (n= 240) and KisKdr (n= 240) were exposed to the insecticides. In control groups, female mortality rates were below 5% (2.5 ± 3.42% for AcerKis) (**Figure 1A**) except in the KisKdr strain (5 ± 4.77%) (**Figure 1B**).

AcerKis resistance was confirmed to both bendiocarb (48.75 ± 10.95%) and chlorpyriphos-methyl (71.25 ± 9.92%) (**Figure 1A**). KisKdr resistance was also confirmed to DDT (2.63 ± 1.05%) and permethrin (42.11 ± 3.44%) (**Figure 1B**).

Reproductive Success

Mosquito Fecundity Following Insecticides Exposure

Females mosquitoes that survived the insecticide susceptibility tests were allowed to lay eggs individually after blood-feeding. AcerKis females laid significantly fewer eggs following exposure to chlorpyriphos-methyl with a median number of eggs per female of 49 eggs compared to that of bendiocarb exposed (82 eggs) and the control (51.5 eggs) mosquitoes (Kruskal-Wallis $\chi^2 = 6.4374$, df = 2, $p = 0.040$) (**Figure 1C**). The percentage of AcerKis females that contributed to the oviposition was higher in bendiocarb exposed (26.83%; $CI_{95\%}$= [14.22 – 42.99]) and in chlorpyriphos-methyl exposed females (21.74%; $CI_{95\%}$= [7.46% – 43.70%]). However, there was no significant difference compared to their control (15.38%; $CI_{95\%}$= [8.21 – 25.33]) (Chi-square $\chi^2 = 2.2944$, df = 2, $p = 0.318$) (**Figure 2**, **Table 1**).

In KisKdr females, were more eggs in individuals who survived DDT exposure (**Figure 1D**). However, no significant difference was observed in the median number of eggs within the different groups (50, 78, 63.5 eggs per female respectively in control, DDT and permethrin exposures; Kruskal-Wallis $\chi^2 = 3.439$, df = 2, $p = 0.179$) (**Figure 1D**). Besides, there was no difference in the percentage of the females that contributed to the oviposition (control: 9.21%, $CI_{95\%}$= [3.79 – 18.06]; DDT 4% exposure: 13.51%, $CI_{95\%}$= [6.68 – 23.45]; permethrin 0.75% exposure: $CI_{95\%}$= [11.47 – 37.84]; Chi-square $\chi^2 = 4.265$, df = 2, $p = 0.118$) (**Figure 2**, **Table 1**).

Mosquito Fertility Following Insecticides Exposure

The egg batches collected from AcerKis and KisKdr females that contributed to the oviposition were allowed to hatch, and the number of larvae was counted. A significant difference in the median number of larvae was observed in AcerKis females following bendiocarb (65 larvae) and chlorpyriphos-methyl (22 larvae) exposure compared to the control females (26 larvae) (Kruskal-Wallis $\chi^2 = 6.9321$, df = 2, $p = 0.031$) (**Figure 3A).** However, there was no significant difference in the hatching rates in the exposed females (67.47 ± 20.42% for bendiocarb and 36.32 ± 16.1% for chlorpyriphos-methyl) compared to the control females (46.92 ± 35.47% (**Figure 3C**).

The results showed a slight increased fertility in KisKdr females exposed to DDT (median larvae per female of 81.91) compared to their control (median larvae per female of 57.89) (Kruskal-Wallis $\chi^2 = 4.221$, df = 1, $p = 0.039$) (**Figure 3B**). As illustrated in **Figure 3D**, the hatching rates were also slightly different within the groups: 60.54%, $CI_{95\%}$ = [44.32 – 76.76] for the control; 77.81%, $CI_{95\%}$ = [68.28 – 87.34] for DDT exposure; and 73.66%, $CI_{95\%}$ = [63.73 – 83.60] for permethrin exposure (Kruskal-Wallis $\chi^2 = 6.355$, df = 2, $p = 0.042$).

DISCUSSION

To date, vector control through the use of insecticides to impregnate mosquito bed nets and dwelling spraying remains

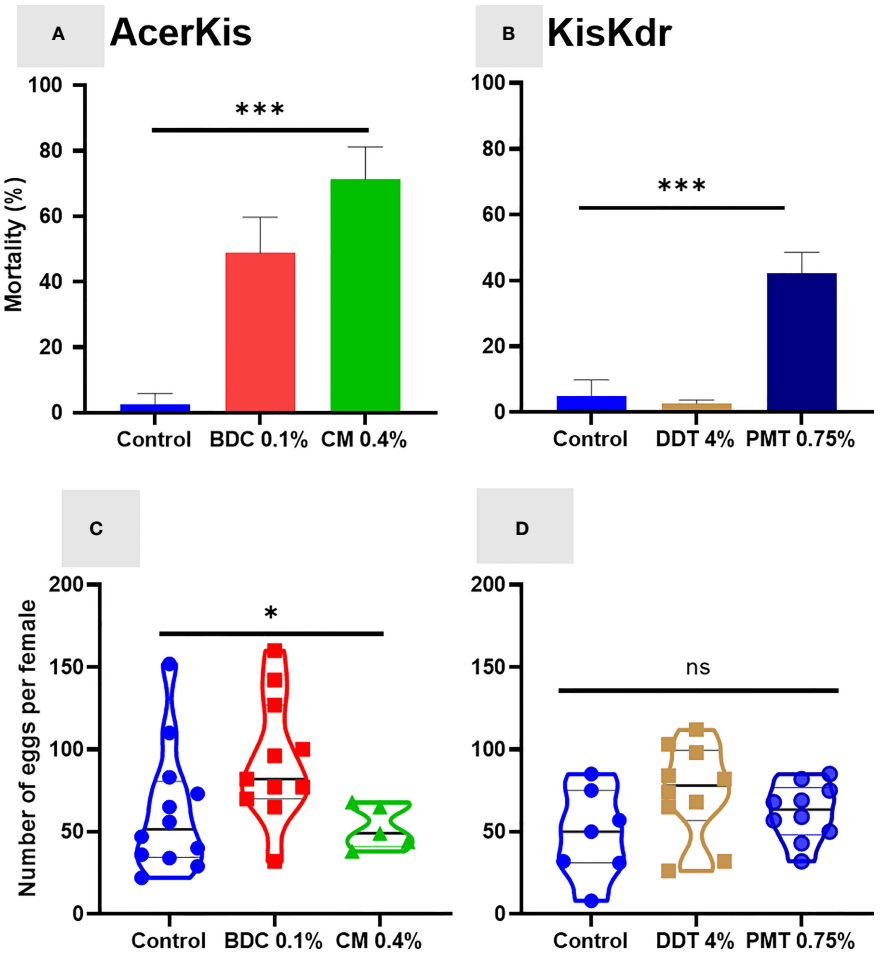

FIGURE 1 | Mortality and fecundity in *An. gambiae* strains following insecticide exposures. Upper panels show *An. gambiae* mortality rates in AcerKis **(A)** and KisKdr **(B)**. Dichlorodiphenyltrichloroethane 4% (DDT 4%) and permethrin 0.75% (PMT 0.75%) exposure time was 3 hours; b endiocarb 0.1% (BDC 0.1%) and chlorpyriphos-methyl 0.4% (CM 0.4%) exposure times were 1 hour and 30 min respectively. All mortality rates were scored 24 hours later. Lower panels show egg production in the same strains: AcerKis **(C)** and KisKdr **(D)**. Each dot represents the number of eggs laid by each female within each group of treatment in each mosquito strain. Only females that laid at least one egg were included for data analysis. Statistical differences were indicated: ns, no significant; ***p value < 0.01; *p value < 0.05. Median numbers of eggs are represented with interquartile ranges. The dots are offset horizontally where overlapping.

the strategy that has significantly contributed to the decline of the malaria burden (2). Nevertheless, due to the increasing insecticide resistance phenomenon, resistant mosquitoes are not instantly killed after their contact with the treated bed nets. However, vectorial capacity could be decreased because of the longer-term impacts of insecticide residues on the mosquito life-history traits that continue after exposure to the impregnated nets (14). The current work investigated the effects of organochlorine (DDT 4%), carbamate (bendiocarb 0.1%), organophosphate (chlorpyriphos-methyl 0.4%), and pyrethroid (permethrin 0.75%) insecticides exposure on *Anopheles gambiae* s.s. fecundity and fertility.

Regarding KisKdr female exposure to permethrin, no significant impact was observed on the fecundity and fertility in this strain. Additionally, only a slight difference in the hatching rate was noticed. These observations imply that permethrin and bendiocarb exposure does not directly affect the reproductive success of the *An. gambiae* mosquitoes carrying kdr^R allele. This knowledge could guide the choice of insecticide compounds when implementing the management programmes of resistant *An. gambiae* mosquitoes to prevent the rapid loss of the effectiveness of the insecticides currently available for vector control. However, the insecticide doses used in our study are the discriminating doses recommended by the WHO (21). They are lower than those used for indoor residual spraying and mosquito nets impregnation. Then, it will be interesting to investigate the effect of direct and long-term exposure to the impregnated mosquito nets on reproductive success using wild-resistant *An. gambiae* mosquitoes. Indeed, Mulatier et al. (22) have already reported that exposure to the bed nets impregnated with permethrin at the WHO recommended dose did not affect fecundity, fertility, or survival of *An. gambiae* laboratory

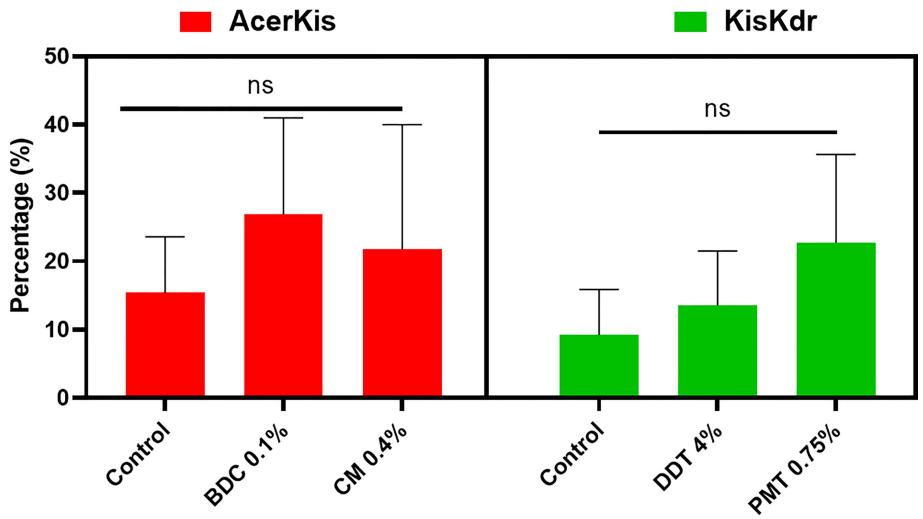

FIGURE 2 | Percentage of females that contributed to the oviposition in each mosquito strain. Dichlorodiphenyltrichloroethane 4% (DDT 4%); permethrin 0.75% (PMT 0.75%); bendiocarb 0.1% (BDC 0.1%) and chlorpyriphos-methyl 0.4% (CM 0.4%). ns, non significant.

colony carrying the kdr^R allele. It is suitable for further works to investigate the long-term effects of insecticides on resistant mosquitoes regarding other life-history traits critical for mosquito vectorial capacity.

Surprisingly, we observed that female mosquitoes carrying the insensitive acetylcholinesterase allele ($ace\text{-}1^R$) that survived after being exposed to chlorpyriphos-methyl laid significantly fewer eggs. This result indicates that the organophosphate compound used might negatively impact the fecundity of homozygous AcerKis female mosquitoes. This would mean that the insecticide hinders either egg development or the ability to lay eggs normally in this mosquito strain. Thus, we assume that the chlorpyriphos-methyl exposure may affect the expression level of key genes involved in egg production and development, such as the *Mating Induced Stimulator of Oogenesis* (*MISO*) (23). Further investigations are needed to understand better which process has led to the egg-laying disruption when exposed to insecticide compounds.

Furthermore, although AcerKis females show resistance to chlorpyriphos-methyl, its impact on the mosquito's fecundity indicates that this insecticide could reduce the vector density (even if the females were not immediately dead) in the long term, reducing malaria transmission. Indeed, natural populations of *Anopheles gambiae* s.l. were already susceptible to pirimiphos-methyl (24) and other organophosphate compounds such as malathion (25, 26) in some African countries. Moreover, wild populations of *Anopheles coluzzii*, a sibling species of *An. gambiae*, have also been shown to be fully susceptible to fenitrothion and pirimiphos-methyl (27).

The presence of alleles that confer insecticide resistance in mosquitoes is correlated to the changes in physiological processes, which drive vector fitness disadvantages (28). For instance, Alout et al. (12), have reported that exposure to pyrethroid insecticides reduced the prevalence and intensity of *Plasmodium falciparum* infection in *Anopheles gambiae* s.s. carrying kdr^R *and* $ace\text{-}1^R$ alleles. It was also reported that *P. falciparum* infection decreases the resistant vector survivorship following insecticide exposure (29). Exposition to pyrethroid insecticide has also been shown to reduce parity in resistant

TABLE 1 | Number of mosquitoes laying eggs and offspring produced in each experimental group.

	AcerKis			KisKdr		
	Control (n=80)	BDC 0.1% (n=80)	CM 0.4% (n=80)	Control (n=80)	DDT 4% (n=80)	PMT 0.75% (n=80)
Numbers of mosquitoes surviving exposure	78	41	23	76	74	44
Number of non-laying females	66	30	18	69	64	34
Number of females laying eggs	12	11	5	7	10	10
Clutch size (min –max number of eggs)	22-152	32-160	38-66	8-85	26-112	32-85
Number of offspring (min –max number of larvae)	3-114	8-117	4-27	4-63	23-100	25-71

Dichlorodiphenyltrichloroethane 4% (DDT 4%); permethrin 0.75% (PMT 0.75%); bendiocarb 0.1% (BDC 0.1%) and chlorpyriphos-methyl 0.4% (CM 0.4%). n, number of mosquitoes in each experimental condition.

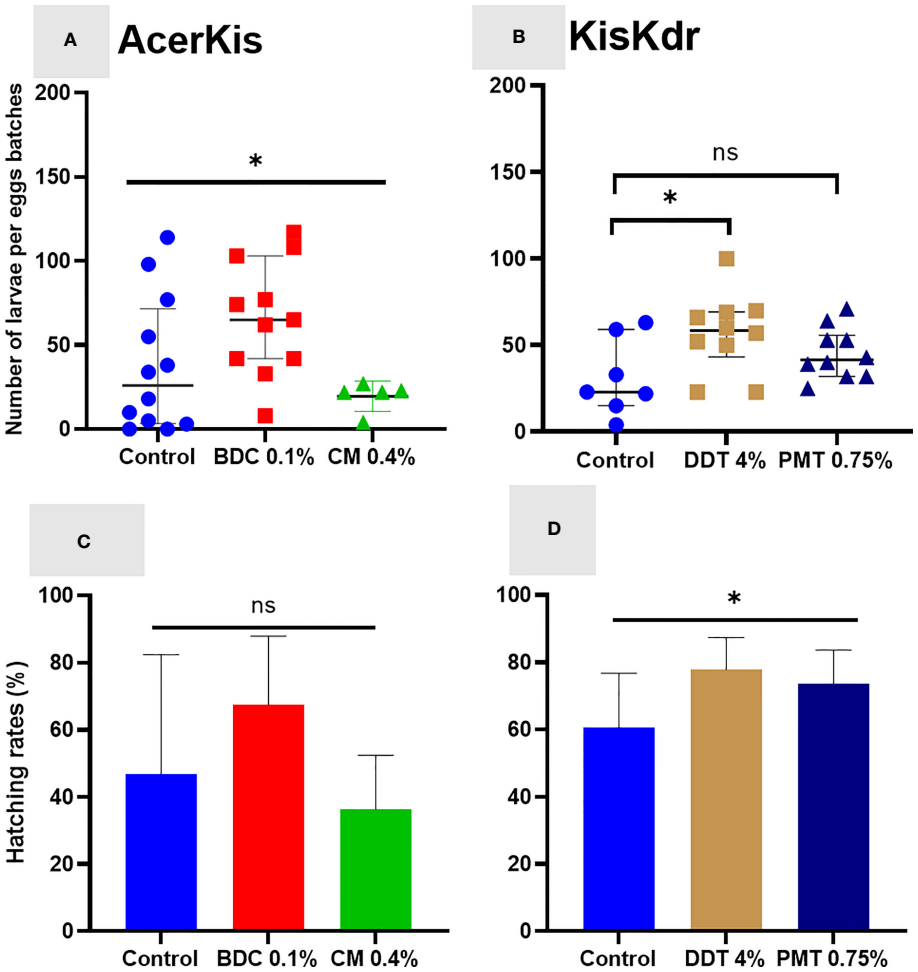

FIGURE 3 | Fertility and hatching rate in *An. gambiae* strains following insecticide exposure. Upper panels show the number of larvae per egg batch in AcerKis **(A)**, and KisKdr **(B)**. Each dot denotes the number of larvae hatched from individual female egg batch within each group of treatment in each strain. Lower panels show the mean larval hatching rates in the same mosquito strains: AcerKis **(C)** and KisKdr **(D)** *p value <0.05; ns, non significant.

Anopheles gambiae and *Anopheles coluzzii* mosquitoes (30). Putting all these observations together, it has become evident that the interaction between insecticide exposure and the mosquitoes' life-history traits can represent a critical aspect of vectors biology that may impact the pattern of malaria parasites transmission and the outputs of vector control measures.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AM, and LSD conceived the study, designed the experiments and revised the manuscript. EBJS, EGS, EA and LD performed the experiments. OD acquired, analysed the data and drafted the manuscript. All authors read and approved the final manuscript.

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