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The Role of Epigenetics in Insects in Changing Environments

The diapausing mosquito *Culex pipiens* exhibits reduced levels of H3K27me2 in the fat body

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Abstract

Culex pipiens, the northern house mosquito, is a major vector of West Nile virus. To survive the severe winter, adult mosquitoes enter a diapause programme. Extended lifespan and an increase in lipid storage are key indicators of diapause. Post-translational modifications to histone proteins impact the expression of genes and have been linked to the lifespan and energy utilisation of numerous insects. Here, we investigated the potential contribution of epigenetic alterations in initiating diapause in this mosquito species. Multiple sequence alignment of H3 sequences from other insect species demonstrates a high conservation of the H3 histone in *Cx. pipiens* throughout evolution. We then compared the levels of histone methylation in the ovaries and fat body tissues of diapausing and non-diapausing *Cx. pipiens* using western blots. Our data indicate that histone methylation levels in the ovaries of *Cx. pipiens* do not change during diapause. In contrast, H3K27me2 levels decrease more than twofold in the fat body of diapausing mosquitoes relative to non-diapausing counterparts. H3K27 methylation plays a crucial role in chromosome activation and inactivation during development in many insect species. This is predominantly governed by polycomb repressor complex 2. Intriguingly, a previous ChIP-seq study demonstrated that the transcription factor FOXO (Forkhead box O) targets the genes that comprise this complex. In addition, H3K27me2 exhibits dynamic abundance throughout the diapause programme in *Cx. pipiens*, suggesting its potential role in the initial activation of the diapause programme. This study expands our understanding of the relationship between alterations in epigenetic regulation and diapause.

KEYWORDS
Culex pipiens, diapause, epigenetics, fat body, H3K27me2, histone methylation

INTRODUCTION

Diapause is an endogenously controlled dormancy that allows some mosquito species to survive adverse environmental conditions such as cold winters. The West Nile virus vector, *Culex pipiens*, enters reproductive diapause in the fall when adult female mosquitoes halt their reproductive maturation, switch from blood feeding to sugar feeding exclusively, migrate to protective sites, enhance cold tolerance, repress metabolic activities and extend longevity (Benoit & Denlinger, 2007; Bowen et al., 1988; Mitchell, 1981; Mitchell & Briegel, 1989; Rinehart et al., 2006). The diapause programme is initiated and maintained by coordinated transcriptional changes in this species. Our previous findings implicate that the reduction in insulin signalling and subsequent higher activity of Forkhead box O (FOXO) is the key molecular switch that activates the diapause programme (Sim & Denlinger, 2008). Subsequently, we found Foxo-target genes using ChIP-sequencing, which regulate many diapause attributes such as overwintering stress tolerance, lifespan extension and metabolic repression (Sim et al., 2015).

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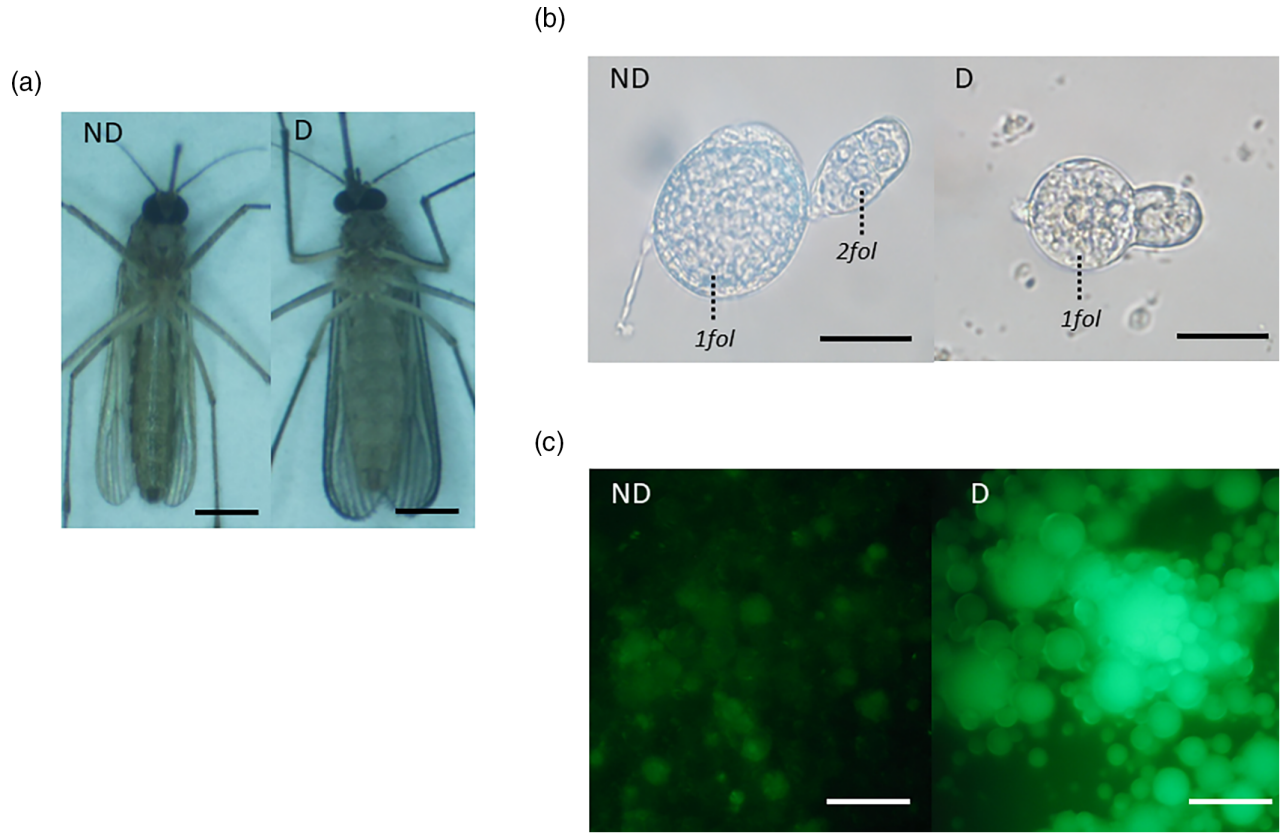

FIGURE 1 Morphological differences in body size, ovarian follicles and fat body cells between non-diapausing (ND) and diapausing (D) adult females of *Culex pipiens* 1 week after eclosion. (a) Significant body size differences between ND females (slim) and D females (fat) of *Cx. pipiens*. (b) Primary (1fol) and secondary (2fol) follicles from ND females and primary follicles from D females, showing the cessation of ovarian development in D females. Both follicles were prepared 10 days after eclosion. Scale bars correspond to 50 μm. (c) Fluorescent microscopy images of fat body cells in D and ND females. Fat hypertrophy was observed in the fat body cells of D females fed on 10% glucose. Lipids in adipocytes were stained using BIODIPY 493/503 (green). White bars represent 100 μm.

In recent years, there have been an increasing number of studies linking epigenetic changes with the phenotypes associated with insect diapause (Bewick et al., 2017; Pegoraro et al., 2016; Werren et al., 2010). By adding methyl groups to DNA's cytosine nucleotides, DNA methylation regulates gene activity. Diapausing wasps display a distinct genome-wide DNA methylation profile compared with non-diapausing wasps, and suppressing DNA methylation altered the diapause response, according to genomics studies of the parasitoid wasp *Nasonia vitripennis* (Pegoraro et al., 2016; Werren et al., 2010). In addition to DNA methylation, the vast majority of eukaryotes use histone post-translational modifications (hPTMs) to control gene expression. Few studies have investigated the potential functions of hPTMs in the control of the diapause programme in *Cx. pipiens*, despite the fact that mosquitoes lack a robust DNA methylation system (Bewick et al., 2017). The basic packing units in the nucleus known as nucleosomes are made of histone proteins and consist of 147 base pairs of DNAs wrapped around a histone octamer (two copies of each of the four core histone proteins H2A, H2B, H3 and H4) (Talbert & Henikoff, 2010). According to Peterson and Laniel (2004), hPTMs are epigenetic signals that are typically found on the N-terminal tails of histone proteins. These influence transcription by either directly affecting histone interactions with underlying DNAs or by enlisting the help of other regulatory factors (effectors) (Ruthenburg et al., 2007; Seet et al., 2006). Histone modifications have been well documented in some insect species and model organisms involved in the biological processes related to diapause. In the fruit fly *Drosophila melanogaster*, heterochromatin marks such as H3K9me2, H3K9me3 and heterochromatin protein 1 (HP1) have significantly lower enrichment in aged flies (Larson et al., 2012). However, lower abundance of the heterochromatin mark H3K27me3 due to a heterozygous mutation in the polycomb repressive complex 2 (PRC2) increased longevity in the same species (Siebold et al., 2010). This indicates that histone modifications have high specificity. *Chymomyza costata* is a drosophilid that enters larval diapause in response to the short-day condition. Genes encoding for an important H3K4 methylation subunit were shown to be down-regulated during diapause in this species (Poupardin et al., 2015). Recently, a study found that H3K4me3 and H3K36me1 are depleted in the ovaries of diapausing *Drosophila* females. RNAi knockdown of the histone modification writers and erasers for H3K4me3 and H3K36me1 altered histone modification levels accordingly and further altered diapause plasticity, demonstrating that depletion of

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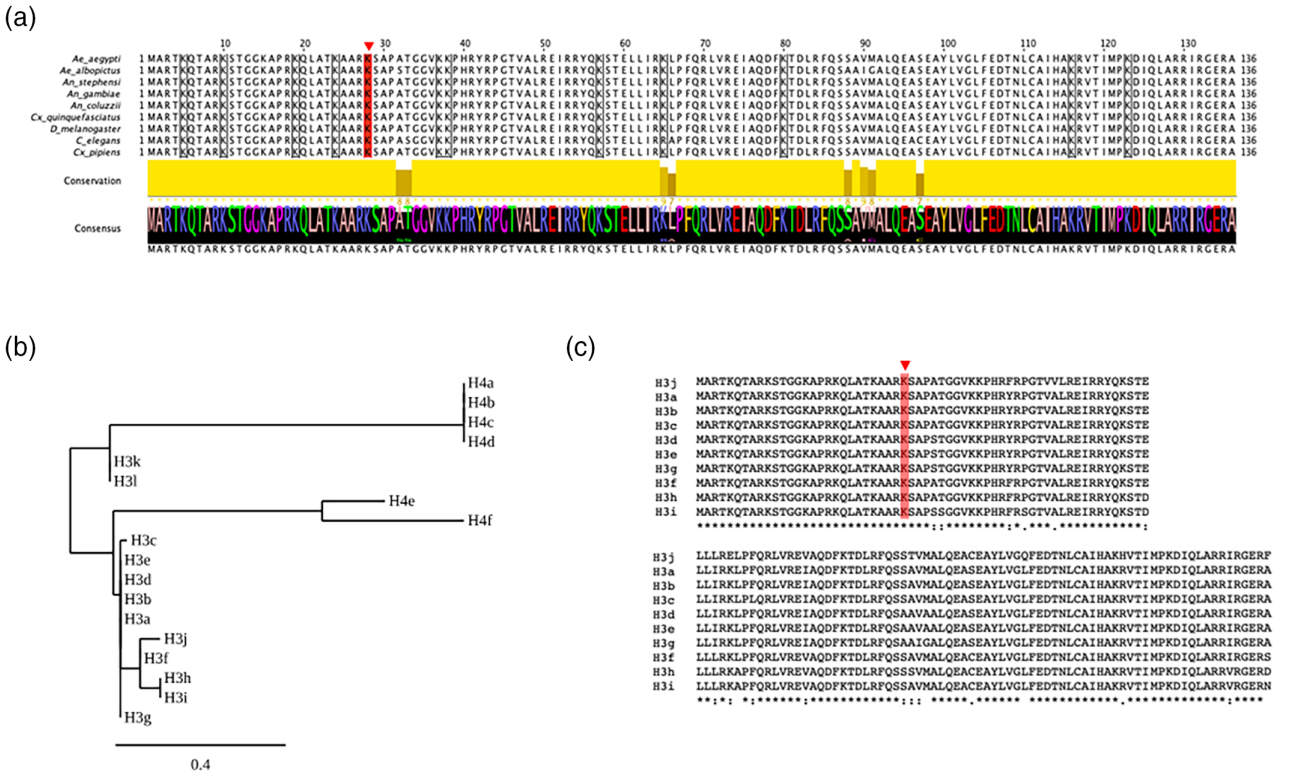

FIGURE 2 Comparison of H3 protein sequences and evolutionary relationships among nine species. (a) Multiple sequence alignment of *Culex pipiens* H3 amino acid sequence with those of *Culex quinquefasciatus* (CQUJHB003479), *Caenorhabditis elegans* (P08898), *Drosophila melanogaster* (FBgn0051613), *Anopheles coluzzii* (ACMO_005058), *Anopheles gambiae* (AGAP003910), *Anopheles stephensi* (ASTE009251), *Aedes aegypti* (AAEL000492) and *Ae. albopictus* (AALF002947). Amino acid positions containing lysine were indicated by black boxes. The 27th lysine on the N-terminus tails of H3 proteins were highlighted in red. Conservation scores (out of 11 with default amino acid property grouping) were visualised by histograms. Consensus sequences represent the relative number of amino acids per column, which is estimated by its size in the logo. (b) Phylogenetic tree of 12 H3 paralogs (H3a, CQUJHB006849; H3b, CQUJHB003479; H3c, CQUJHB015331; H3d, CQUJHB017365; H3e, CQUJHB001464; H3f, CQUJHB004173; H3g, CQUJHB005320; H3h, CQUJHB006332, H3i, CQUJHB006695; H3j, CQUJHB001229; H3k, CQUJHB015627; H3l, CQUJHB008015) and six H4 paralogs (H4a, CQUJHB017511; H4b, CQUJHB001516; H4c, CQUJHB006644; H4d, CQUJHB006040; H4e, CQUJHB000447; H4f, CQUJHB005776) in *Cx. quinquefasciatus*. (c) Multiple sequence alignment of *Cx. pipiens* H3 paralogs (H3a–H3j). The 27th lysine on the N-terminus tails of H3 proteins were highlighted in red and red arrow head. "*" indicates amino acids that are identical in all sequences in the alignment.

H3K4me3 and H3K36me1 promotes diapause plasticity (Evans et al., 2022). Studies linking histone modifications and insect diapause drive us to explore the possibility that histone methylation may play a role in orchestrating the transcriptional switches that initiate the diapause programme in *Cx. pipiens*.

Thus far, limited evidence exists that points to histone methylations as a diapause regulator in *Cx. pipiens*. Here, we examine the potential regulatory effect of histone methylation in initiating the diapause programme in *Cx. pipiens*. We collected lysate from the fat bodies and ovaries of both diapausing and non-diapausing mosquitoes and screened nine histone H3 modifications that are present in high abundance in order to identify the histone modifications that are associated with diapause initiation. We provide evidence that H3K27me2 levels are significantly lower in the fat body of diapausing mosquitoes. We also identified unique changes in the level of methylation at this histone site in the fat body of mosquitoes throughout the diapause programme. Together, these results suggest that H3K27me2 may play a key role in mediating early diapause programme in *Cx. pipiens*.

RESULTS

Confirmation of diapausing phenotypes in female *Cx. pipiens*

To confirm the diapause status of mosquitoes, we compared the morphological differences between diapausing (D) versus non-diapausing (ND) adult female mosquitoes 1 week after eclosion. The adult females reared under the short-day condition (9 h:15 h light:dark [L:D]) showed enlarged abdomens compared with long-day-reared ND females (15 h:9 h L:D) (Figure 1a). In addition, while ovarian development was robust in ND females 1 week after adult eclosion, primary and secondary follicles were not developed in D females (Figure 1b). Both groups were kept at 18°C and 75% relative humidity with access to 10% sucrose solution. The abdomens of D and ND female mosquitoes were dissected and the microscope images of BODIPY 493/503 stained fat body cells from the D females showed an increase in the total amount of lipids found in fat bodies compared with their control ND counterparts (Figure 1c).

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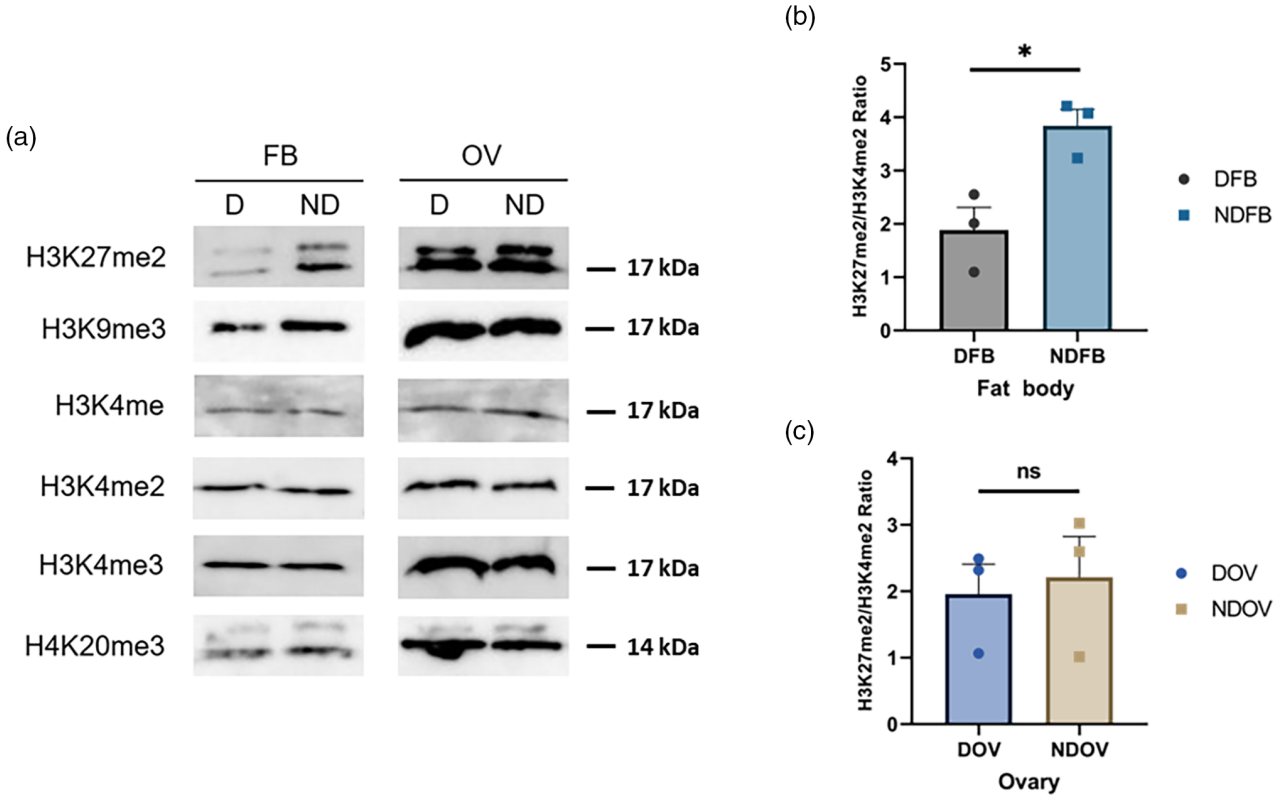

FIGURE 3 Different expression levels of methylated histone protein abundance in *Culex pipiens* female tissues 1 week after adult eclosion. (a) Diverse methylated histones were detected in tissues from adult females, including fat bodies (FB) and ovaries (OV). (b) H3K27me2 protein levels in the fat body were normalised to protein levels of H3K4me2 ($n = 3$, bar = SE). The level of H3K27me2 protein abundance in the fat body of D females was significantly different from all others (*$p < 0.05$) by Student's *t*-test. (c) H3K27me2 protein levels in the ovary were normalised to protein levels of H3K4me2 ($n = 3$, bar = SE). There was no significant difference between the H3K27me2 level in D and ND ovaries.

H3 protein is highly conserved among mosquito species and model organisms

To validate the conservation of H3 proteins among mosquito species and other model organisms, we examined the amino acid sequences of H3 proteins from seven closely related mosquito species: *Aedes aegypti*, *Aedes albopictus*, *Anopheles stephensi*, *Anopheles gambiae*, *Anopheles coluzzi*, *Culex quinquefasciatus*, *Cx. pipiens* and two model organisms: *D. melanogaster* and *Caenorhabditis elegans* from Vectorbase and Uniprot. We then performed multiple sequence alignment using these sequences (Figure 2a). All sequences are 136 amino acids in length. *Cx. pipiens* H3 sequences matched 100% with those of all other species except for *Ae. albopictus* and *C. elegans*. *Cx. pipiens* H3 also has high conservation with *C. elegans* and *Ae. albopictus* by having a 97% sequence identity. The 27th lysine on the N-terminal tail is conserved among all nine organisms (Figure 2a). In addition, the 12 H3 and 6 H4 paralogs in *Cx. quinquefasciatus* were selected for the phylogenetic analysis to infer the evolutionary relationships between H3 and H4 proteins in *Cx. pipiens* (Figure 2b,c). The majority of the H3 and H4 amino acid sequences are clustered together, indicating high conservation evolutionarily (Figure 2b). Multiple sequence alignment of H3 paralogs showed 86% of the total sequences are common in all paralogs (Figure 2c), further suggesting that H3 protein is highly conserved in this species.

H3K27me2 is significantly lower in the fat body cells of diapausing mosquitoes

To identify the unique histone methylation modification(s) in diapausing adult *Cx. pipiens*, we examined the level of nine histone methylation modifications from the fat body and ovary samples in both diapausing and non-diapausing mosquitoes 7 days post-eclosion by western blot (Figure 3a). Within those nine histone methylation modifications, three modifications failed and did not show any signals through western blot (H3K27me3, H3K36me3 and H3K27me1). The western blot analysis demonstrated that although majority of histone methylation modifications showed no difference between diapausing and non-diapausing mosquitoes (Figure S1), antibody directed against H3K27me2 detected significantly lower abundance in the diapausing fat body tissues compared with those of non-diapausing counterpart. Quantification of western blot bands showed that the difference between ND and D for H3K27me2 in fat body samples was more than twofold ($p < 0.01$, $n = 3$) (Figure 3b) (Table S1). No significant difference was shown from the quantification of western blot bands between D and ND ovary samples (Figure 3c). Our H3 antibody failed to show strong signals, thus we used H3K4me2 as a loading control since H3K4me2 showed consistent levels of abundance in both diapausing and non-diapausing conditions during our initial western blot testing (Figure 3a) (Table S1).

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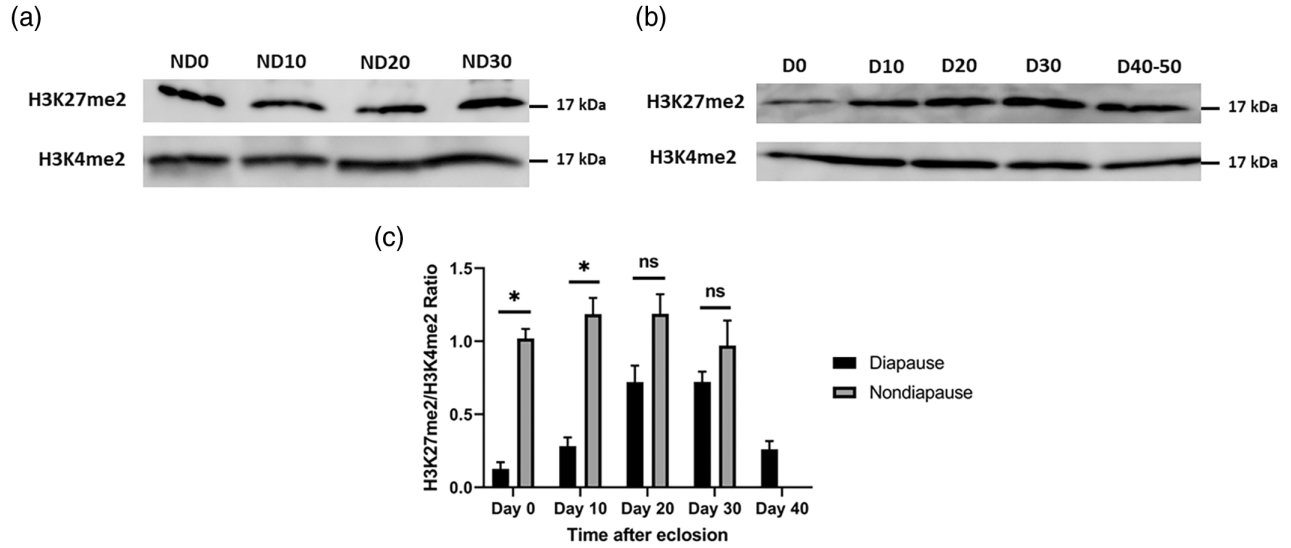

FIGURE 4 Temporal patterns of the H3K27me2 protein abundance in the different stages of diapause. ND represents adult females reared under non-diapausing conditions. (a) Western blot showing H3K27me2 levels in the fat body of ND female *Culex pipiens* 0, 10, 20 and 30 days post-eclosion. (b) Western blot showing changes in H3K27me2 levels in the fat body of D female *Cx. pipiens* 0, 10, 20, 30, 40 and 50 days post-eclosion. H3K4me2 was used as a loading control. (c) Comparison of H3K27me2 abundance levels between the fat bodies of D and ND mosquitoes at different time points after eclosion. H3K27me2 abundance levels between ND and D were normalised to protein levels of H3K4me2 ($n = 3$, bar = SE). The abundance of H3K27me2 protein in the fat body from the early diapausing period was significantly lower than in the counterpart ND fat body cells (*$p < 0.05$) by *t*-test with Bonferroni correction. ns = not significant.

H3K27me2 level is dynamic during the diapause programme in the fat body

In order to measure the change in H3K27me2 in early and late diapause periods, samples were extracted by time period from the time of adult eclosion to the time of late diapause progressing—about 40–50 days after adult eclosion. Also, comparisons were made with non-diapause samples at the respective times. However, since non-diapausing mosquitoes cannot live up to 40–50 days, non-diapause samples at this time were excluded from the comparison. We collected fat body histone samples from ND mosquitoes at four time points: right after eclosion (ND0), 10 days post-eclosion (ND10), 20 days post-eclosion (ND20) and 30 days post-eclosion (ND30). Western blot analysis performed with ND samples revealed that H3K27me2 was consistently up-regulated in the fat body of ND mosquitoes (Figure 4a) ($n = 3$). We extracted D fat body histone samples at five time points: right after eclosion (D0), 10 days post-eclosion (D10); 20 days post-eclosion (D20); 30 days post-eclosion (D30); and 40–50 days post-eclosion (D40–50). Western blot analysis of D samples indicated that H3K27me2 levels fluctuate during diapause programme (Figure 4b,c). To compare the H3K27me2 abundance in both D and ND timepoints, we performed a two-way analysis of variance (ANOVA). The independent variables for the analysis were the diapause conditions and time after eclosion, while the dependent variable was the abundance of H3K27me2 methylation. Our analysis using the two-way ANOVA test revealed a significant difference in both the D and ND comparisons and among different time points after eclosion ($p < 0.05$). By conducting post hoc tests to evaluate the differences among means within diapause and non-diapause samples, we observed that no statistical significance was found within non-diapause samples. However, the abundance of H3K27me2 levels showed significant differences on day 0, day 10 and days 40–50 compared with day 20 and day 30 samples, as determined by Tukey's multiple comparison test (Tables S2 and S3). The H3K27me2 abundance levels from early diapausing females (D0 and D10) were significantly lower compared with those of the ND counterparts, as determined by Bonferroni's multiple comparisons test ($p < 0.05$, Table S4). The increasing trend of H3K27me2 abundance stopped after D20 and there were no significant changes after D20 (Table S4).

DISCUSSION

Diapause provides insects with a different developmental pathway so they can endure harsh seasons. Previous studies have shown that various diapause phenotypes might be regulated by epigenetic modifications in some insect species (Meister et al., 2011; Pegoraro et al., 2016; Yocum et al., 2015). H3K27me2/3 are heterochromatin histone modifications associated with repressive chromatin state. In the nematode *C. elegans* and fruit fly *D. melanogaster*, histone methyltransferases/demethylases regulating these histone modifications have been illustrated to play an important role in lifespan extension, which is also a key diapause trait in mosquito *Cx. pipiens* (Jin et al., 2011; Ma et al., 2018; Maures et al., 2011; Ni et al., 2012; Siebold et al., 2010). To date, no research has investigated the potential effects of histone modifications on diapause phenotypes in

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Cx. pipiens. In this study, we attempted to discover novel histone modifiers that modulate diapause characteristics in *Cx. pipiens*. We examined the levels of nine different histone modifications and found H3K27me2 levels to be significantly lower in the fat body of diapausing adult females. In addition, we measured the change in the abundance of H3K27me2 modification for each period of diapausing and non-diapausing adult females.

Histone H3 is an evolutionarily conserved histone protein (Delange et al., 1969; Wells & Brown, 1991). To confirm this in mosquitoes, we extracted and aligned amino acid sequences of H3 protein from seven mosquito species along with two model organisms. We found H3 to be highly conserved throughout evolution among mosquitoes and the model species, such as the fly and nematode. We also confirmed very high conservation of 10 H3 paralogs from *Cx. quinquefasciatus* through a phylogenetic analysis. This high level of amino acid sequence conservation persists evolutionarily and among paralogs within a species, which suggests that H3 protein has an essential function for the survival of an organism and a high functional similarity among diverse species.

Transcriptional profiling analysis from several insect species has implicated that epigenetic regulations, especially histone methylations, regulate diapause features (Meister et al., 2011; Poupardin et al., 2015). For example, *dpy-30* encodes for a H3K4 methyltransferases and was down-regulated in the drosophilid fly, *Chymomyza costata*, which was reared in diapausing condition; biological processes controlled by DPY-30 are arrested during diapause (Poupardin et al., 2015). In our previous work, mRNA expression of Pax-interacting protein (Pax), a prominent diapause modulator in *Cx. pipiens* downstream of FOXO, is up-regulated fourfold in diapausing *Cx. pipiens* (Sim et al., 2015). Pax plays a role in the localization of Sirtuin 1 (Sir2), an NAD-dependent histone deacetylase that regulate lifespan extension in fruit fly and roundworm (Latham & Dent, 2007; Lechner et al., 2000; Rogina & Helfand, 2004; Tissenbaum & Guarente, 2001). This observation suggests that histone deacetylation may at least in part be involved in the increase of the lifespan in diapausing females.

In addition, we found that H3K27me2 levels are significantly decreased in the fat body of diapausing mosquitoes. H3K27me2/3 are heterochromatin modifications that have been associated with repressive chromatin state (Czermin et al., 2002; Lee et al., 2015; Müller et al., 2002). Core members of polycomb repressive complex 2 (PRC2) are responsible for the formation of H3K27me2/3 modifications (Schuettengruber & Cavalli, 2009; Simon & Kingston, 2009). Polycomb group (PcG) proteins are well conserved evolutionarily, and they silence gene expression by inducing chromatin structure remodelling through the formation of PRC1 and PRC2. PRC2-mediated H3K27 methylations have been implicated in many key developmental processes such as developmental timing (Lu et al., 2013; Yang et al., 2021), glycolysis and lifespan extension (Ma et al., 2018; Siebold et al., 2010). In the cotton bollworm *Helicoverpa armigera*, extra sex comb (ESC)—a member of PRC2—is up-regulated in ND individuals. RNAi against the gene encoding ESC reduces H3K27me2/3 levels, which inhibits the activity of the prothoracicotropic hormone (PTTH) and, eventually, develops a diapause-like symptom (Lu et al., 2013). Additionally, *utx* encodes for a histone demethylase (KDM) that removes all methylations on the 27th lysine of H3 (H3K27) (Hong et al., 2007). RNAi knockdown against the gene encoding KDM in *C. elegans* elevated H3K27me2/3 levels and extended the lifespan (Jin et al., 2011; Maures et al., 2011), a result contradicting with previous studies where reduced H3K27 methylations extended the lifespan in *Drosophila* (Ni et al., 2012; Siebold et al., 2010). Therefore, the effect of this methylation on longevity is likely distinct among different species. However, it is not well known at present by what molecular mechanisms histone methylation regulates longevity among diverse organisms. Notably, the impact of H3K27 methylations on lifespan extension is dependent on the insulin/FOXO signalling pathway (Jin et al., 2011; Ni et al., 2012). Maures et al. (2011) found that knocking down *utx-1*, the gene encoding for a H3K27me2/3 histone demethylase, extended the lifespan of *C. elegans*. However, *utx-1* knockdown did not extend the lifespan of the *daf-16* (*foxo*) mutant worms, thus suggesting that altering H3K27me2/3 levels regulates longevity by modulating the insulin-FOXO signalling pathway in *C. elegans*. We previously identified FOXO to be the master regulator of the diapause programme in *Cx. Pipiens*, downstream of insulin signalling pathway (Sim & Denlinger, 2008). Thus, we propose that H3K27me2 contributes to the extension of lifespan in diapausing female *Cx. pipiens*, at least in part, through the interaction with insulin/FOXO signalling pathway. While H3K27me2 levels appeared to be differentially maintained between D and ND fat body, we failed to detect any significant differences in the levels of histone modifications between D and ND ovary in our western blot study. We speculate that the phenotypic changes that occur in the ovary during diapause are a result of other epigenetic modifications such as histone acetylation and DNA methylation. *Anopheles albimanus*, a key vector for *Plasmodium vivax*, has a functional DNA methylation system that is responsive to larval viability and parasite challenge (Claudio-Piedras et al., 2020). Therefore, further research should focus on other aspects of epigenetic regulation in *Cx. pipiens*.

The diapause programme typically proceeds through three characteristic phases: pre-diapause, diapause, and post-diapause (Koštál, 2006). During the preparation and initiation phases of insect diapause, transcriptions of genes related to building energy reserves up-regulate to prepare for diapause maintenance when development and metabolism halt. In *Cx. pipiens*, diapause evokes changes in molecular machinery involved in energy metabolism. Genes encoding carbohydrate metabolism and fat accumulation are actively transcribed in diapausing females. In our previous study, we explored the expression patterns of 31 fat-related genes throughout diapause and found that genes related to lipid accumulation, such as *fatty acid synthase-1*, *fas-3* and *fatty acid-binding protein* are significantly elevated during early diapause. On the contrary, genes related to energy metabolism were down-regulated in early diapause (Sim & Denlinger, 2009). *Glycogen synthase* is involved in glycogen and lipid accumulation in early diapause (King et al., 2020). Moreover, genes related to stress tolerance mechanisms are also promoted during diapause initiation. Knocking down the expression of stress tolerance gene *oxidor* in early diapausing *Cx. pipiens* caused a shortened lifespan for diapausing mosquitoes (King et al., 2021). In the current study, we

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investigated the level of abundance of H3K27me2 during the diapause programme; a western blot analysis showed the levels of H3K27me2 to be significantly lower in diapausing mosquitoes on day 0 and day 10 post-eclosion relative to their ND counterpart; the levels remained consistent throughout the rest of diapause. Given that most of the active transcriptions occur in early diapause, the results of this study suggest that the loss of the H3K27me2 modifications may play an important role in activating specific genes and eventually initiating the diapause programme. Therefore, determining which genes are regulated by H3K27me2 during diapause is of interest for future studies.

In conclusion, we performed a small-scale screening of differential histone methylation patterns in the fat body and ovary cells of both diapausing and non-diapausing *Cx. pipiens* adults. We demonstrated that the H3K27me2 modification is significantly lower in the fat body of diapausing female mosquitoes. Other studies have reported that PRC2-mediated H3K27 methylations, working cooperatively with UTX-induced H3K27 demethylations, are responsible for an extensive number of developmental processes, many of which are associated with diapause features such as developmental timing, longevity, stress resistance and glycolysis (Jin et al., 2011; Lu et al., 2013; Ma et al., 2018; Maures et al., 2011; Ni et al., 2012; Siebold et al., 2010; Yang et al., 2021). Thus, our results point to this histone modification as a promising regulator for the unique diapause phenotypes in *Cx. pipiens*. Future studies should also characterise the potential roles of histone modifiers (PcG proteins and KDMs) and how they regulate specific phenotypes in this species.

EXPERIMENTAL PROCEDURES

Mosquito rearing

The *Cx. pipiens* colony was established in September 2000 from larvae collected in Columbus, OH; additional field-collected mosquitoes from Dr. Meuti's laboratory at Ohio State University were added to the colony in 2009 and 2022. The *Cx. pipiens* colony was reared under a 15 h:9 h L:D photoperiod at 25°C and 75% relative humidity (RH) as previously described (Sim & Denlinger, 2008). Hatched larvae from blood-fed adult females were transferred into plastic trays containing distilled water at a density of 300 individuals per tray. Larvae were provided with TetraMin® fish flakes and pupae were placed in a water cup inside a cage with 10% sucrose solution for eclosed adults. To induce diapause, mosquito larvae were transferred at second-instar stage to an 18°C chamber with 75% RH and 9 h:15 h L:D photoperiod. Diapause status was confirmed by measuring primary follicle and germarium lengths, and ovarian development stage was determined based on described methods (Christophers, 1911).

Measurements of fat body tissues and follicles

Fat body tissues and follicles were dissected from diapausing and non-diapausing females 1 week after adult eclosion. Tissues were placed in a drop of saline solution, dissected with a needle, and examined at 200- and 400-fold magnifications (Zeiss Axioskop, Thornwood, NY). Fat body cells were examined by staining fixed tissues with BODIPY 493/503 (Invitrogen), which was diluted in PBS to a concentration of 1 mg/mL. Stained fat body was examined with a Zeiss Axioskop fluorescent microscope. In addition, follicles were then analysed with an Olympus SZH-ILLD light microscope with an attached DP72 12.8-megapixel digital camera and DP2-TWAIN software (Olympus Corp, Center Valley, PA). Lengths of 10 follicles were calculated for 11–12 individuals. A Student's *t*-test was used to distinguish differences in the sizes of these two tissues in diapausing and non-diapausing females.

Identification and bioinformatics analysis of mosquito H3 protein

Genes encoding for *Cx. pipiens* H3 proteins were used in discontinuous MEGA-BLAST searches on trace archives of genome data from the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with the identity of H3 proteins from other mosquitoes confirmed by performing BLASTN against the Vectorbase (https://vectorbase.org/vectorbase/app/). The protein sequences of H3 proteins from Culex species were aligned with the amino acid sequences of H3 homologues from other mosquitoes and fruit flies using a Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic and molecular evolutionary analyses were conducted by the maximum likelihood method in MEGA X software, with bootstrap values being calculated from 1000 trees. The percentage of replicate trees in which the associated H3 clustered together in 1000 bootstrap replicates was shown adjacent to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Western blot analysis of nine H3 methylation sites in diapausing and non-diapausing females

The protein abundance pattern of nine histone methylation patterns was analysed by western blot. Protein extracts were prepared from the fat body and ovary of two female groups 7–10 days after adult eclosion: these two experimental groups were reared either under 18°C, 75% RH, 9 h:15 h L:D to initiate programme for diapause or under 18°C, 75% RH, 15 h:9 h L:D for the non-diapause control group. Histone protein samples were collected using a histone extraction kit in accordance with the manufacturer's instructions (Abcam, MA, USA). Each sample used 30 fat bodies and 50 ovaries. Three replicates were tested. Protein quantifications were confirmed with Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific). Samples were then centrifuged at 16,800***g*** for 5 min and an equivalent volume of 2× loading buffer (200 mM Tris, 10% SDS, 50% glycerol 400 mM dithiothreitol, 0.1% Coomassie Blue) was added to the supernatant. Samples were heated to 95°C for 5 min before loading per lane on a

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10% SDS gel. In each well, 10 g of protein samples were loaded. The proteins were transferred to a polyvinylidene fluoride membrane (Whatman, Florham Park, NJ, USA) and blocked with 5% low-fat dried milk in tris-buffered saline (TBS) and 0.2% Tween 20 overnight at 4°C. The primary antibodies against H3K27me3 (Millipore, 07-449), H3K27me2 (Abcam, ab24684), H3K27me (Millipore, 07-448), H3K9me3 (Abcam, ab8898), H3K4me3 (Millipore, 07-473), H3K4me2 (Millipore, 07-030), H3K4me (Millipore, 07-436), H3K36me3 (Abcam, ab9050) and H4K20me3 (Abcam, ab9053) were diluted in blocking solution (1:5000 dilution) and incubated at room temperature (RT) for 2 h. The membrane was then washed three times for 30 min at RT with TBS with 0.2% Tween 20 (TBST) followed by incubation with an anti-rabbit-HRP secondary antibody (1:1000 dilution; Cell Signalling Technology, Beverly, MA, USA) for 2 h at RT. The membrane was washed three times with TBST for 30 min at RT followed by LumiGLO chemiluminescent reagent (KPL; Gaithersberg, MD, USA). Each treatment was replicated at least three times. Changes in protein levels were analysed with ImageJ and were normalised to control immunoblots and expressed as fold-change relative to controls. H3K4me2 was used as the loading control as it showed consistent abundance in all biological replicates in both ovary and fat body samples.

Time series analysis of the H3K27me2 protein

Histone protein samples of diapausing female mosquito's fat bodies were extracted from five different time points: 0-day post-eclosion, 10-day post-eclosion, 20-day post-eclosion, 30-day post-eclosion and 40–50-day post-eclosion. Histone protein samples of ND female mosquito's fat bodies were extracted from four different time points: 0-day post-eclosion, 10-day post-eclosion, 20-day post-eclosion and 30-day post-eclosion. Changes in histone methylation levels were examined through the western blot using a primary antibody against H3K27me2. ImageJ was used to analyse and normalise protein bands.

Statistical analyses

The difference between histone methylation abundance between D and ND samples were evaluated using Student's *t*-test. A two-way ANOVA was performed to evaluate the differences in H3K27me2 methylation abundance between D and ND, as well as time after eclosion. The abundance changes in H3K27me2 between different time points within D and ND samples were evaluated using post hoc Tukey's multiple comparisons test. To compare the differences among means between diapause versus non-diapause at different time points, we conducted Bonferroni's multiple comparisons test following the two-way ANOVA analysis.

AUTHOR CONTRIBUTIONS

Xueyan Wei: Conceptualization; methodology; software; data curation; investigation; validation; formal analysis; visualization; writing – original draft; writing – review and editing. **Prabin Dhungana:** Investigation; validation; formal analysis; software. **Cheolho Sim:** Conceptualization; methodology; writing – original draft; writing – review and editing; resources; supervision; project administration; funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

This research was conducted on invertebrate species that were not subjected to any specific ethical issue or legislation.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Abundance levels of histone methylation marks in fat bodies and ovaries of diapausing and non-diapausing *Cx. pipiens* 1 week after adult eclosion. Protein levels of histone methylation marks were normalised to protein level of H3K4me2 except for H3K4me2

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(normalised to H3K4me3) ($n = 3$, bar = SE). Student's *t*-tests showed no significant differences between all abundance levels in D and ND mosquitoes.

Table S1. Statistical results from the comparisons of H3K27me2 levels between diapausing and non-diapausing mosquitoes.

Table S2. Statistical result of post-hoc Tukey's multiple comparisons test within diapause.

Table S3. Statistical result of post-hoc Tukey's multiple comparisons test within non-diapause.

Table S4. Statistical result of post-hoc Bonferroni's multiple comparisons test comparing diapause versus non-diapause at different time points.

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