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Suppressed expression of oxidoreductin-like protein, *Oxidor*, increases follicle degeneration and decreases survival during the overwintering diapause of the mosquito *Culex pipiens*

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ABSTRACT

Throughout diapause in mosquitoes, stress resistance and subsequent prolonged lifespan are a few important features of diapause that are crucial for overwintering success. In the mosquito *Culex pipiens,* we suggest that oxidoreductin-like protein is involved with these diapause characteristics for overwintering survival. Expression of *oxidor* was more than two-fold higher in early stage diapausing females compared to their non-diapausing counterparts. Suppression of the gene that encodes oxidoreductin-like protein by RNAi significantly increased the proportion of degenerating follicles in early-stage adult diapausing females. Inhibition of *oxidor* also significantly reduced the survivability of diapausing females which indicates that this protein plays a key role in protecting multiple tissues during early diapause.

1. Introduction

Organismal lifespan is a complex process that relies on the combination of environmental and genetic factors. In the nematode *Caenorhabditis elegans* and the fly *Drosophila melanogaster,* genetic screens identified the insulin/forkhead transcription factor as a critical signaling pathway that regulates their lifespans. Interestingly, many of the genes found in the screening for extended lifespan are the same as those identified in studies of diapause (Yamamoto and Tatar, 2011; Lee et al., 2001). In several ways, diapause is a considerable extension of an organism's lifespan (Sim and Denlinger, 2008). Studies throughout the years have well documented data on distinct adult diapause in *Cx. pipiens* (Mitchell, 1983; Mitchell and Briegel, 1989; Sanburg and Larsen, 1972; Sim et al., 2015; Kang et al., 2021). Diapause plays an important role in helping this mosquito survive the cold winter months. Other subspecies such as *Culex p. quinquefaciatus* do not have the characteristics of overwintering diapause, so *Cx. p. quinquefaciatus* cannot survive in areas with cold winters. In addition, only females can overwinter through diapause in the mosquito *Culex. p. pipiens (hereafter Cx. pipiens)*. Therefore, the number of mosquitoes that transmit the diseases such as West Nile virus during the winter decreases most significantly during the year. Currently, genetic manipulation through the rapidly advanced CRISPR/Cas9 technology is actively being studied in this mosquito (Feng et al., 2021; Anderson et al., 2019). If we know which genes play an important role in the winter survival of the mosquito *Cx. pipiens,* it is expected that this method can be applied to population control that inhibit the overwintering diapause mechanism.

Recent studies on the molecular regulation of diapause in *Cx. pipiens* show the importance of insulin and forkhead transcription factor (FOXO) as crucial elements in the signaling pathway that leads to the diapause phenotype. In short, the insulin signaling pathway is not activated in diapausing adult females, therefore lifting the suppression of FOXO. Upon this expression of FOXO, the induction of target genes associated with diapause, including enhanced stress tolerance, extended lifespan, and accumulation of fat reserves are then observed (Sim and Denlinger, 2009, 2011; Sim et al., 2015). This mechanism of diapause is conserved in some species, as shown from results with the nematode *C. elegans* and the fly *D. melanogaster* (Giannakou and Partridge, 2007;

Abbreviations: Oxidor, oxidoreductin-like protein; *dsi-oxidor*, dicer-substrate RNAi of *Cx. pipiens* oxidoreductin-like protein; *dsi-control*, dicer-substrate RNAi of control gene; qRT-PCR, quantitative real-time PCR; RNAi, RNA interference; ND, non-diapause; D, diapause..

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Lee et al., 2001). Enhanced stress tolerance is one of the hallmarks of diapause (Denlinger, 2002), and several genes encoding stress-related proteins were revealed in the ChIP-seq analysis as being under the control of FOXO. One is an *oxidor*, the gene encoding oxidoreductin-like protein, a protein likely involved in reducing oxidative stress during mosquito diapause. Catalase and superoxide dismutase, enzymes involved in the detoxification of reactive oxygen species (ROS), are also activated in early diapause (Sim and Denlinger, 2011), which further underscore the importance of the stress response during diapause and suggest that diapausing mosquitoes effectively avoid oxidative damage that can accumulate over winter. Thus, *oxidor* is possibly link to the increased lifespan of diapausing mosquitoes.

Oxidor is homologous to the ERO1 protein in other model organisms, and in previous studies, oxidoreductin-like protein shows enzyme activity that catalyzes the transfer of electrons from one molecule to another by catalysis of an oxidation-reduction reaction where a sulfur-containing group acts as a hydrogen or electron donor and reduces disulfide (Seo et al., 2015; Kim et al., 2012). This suggests that oxidoreductin-like protein metabolizes reactive oxygen species and induces proper protein folding, and if suppressed, inhibition of this stress response occurs. In this study, we propose that this antioxidant enzyme contributes considerably to the extended lifespan and increased stress tolerance of the diapausing mosquito, *Cx. pipiens*. This gene was cloned from *Cx. pipiens* and RNA interference was used to evaluate the function compared between diapausing females and their non-diapausing counterparts.

2. Materials and methods

2.1. Mosquito rearing

The *Culex pipiens pipiens* colony was established in September 2000 from larvae collected in Columbus, OH, and additional field-collected mosquitoes were added to the laboratory colony in 2009. The colony was reared at 25 °C and 75% relative humidity (RH) under a 12:12 Light-Dark (LD) photoperiod, as previously described (Robich and Denlinger, 2005). When larvae reach their first or second instar stage, rearing containers were placed under one of two environmental conditions: nondiapausing adults were reared at 18 °C, 75% RH, and 15 L:9D h (ND), and to induce diapause, mosquitoes were reared at 18 °C, 75% RH, and 9 L:15D h (D). To confirm diapause status, primary follicle and germarium lengths were measured, and the stage of ovarian development was determined according to methods previously described (Christophers, 1911). Larvae were reared in dechlorinated tap water and fed Tetramin fish food (Tetra Holding Inc., Blacksburg, VA). Adults were maintained on honey-filled vials with soaked wicks and kept in 33.5 cm × 30 cm × 30 cm screened cages.

2.2. Indentification and bioinformatics anaylsis of mosquito oxidoreductin-like protein

Cx. pipiens gene encoding oxidoreductin-like protein (*oxidor*) was utilized in discontinuous MEGA–BLAST searches on trace archives of genome data from the NCBI database (http://www.ncbi.nlm.nih.gov/blast/ tracemb.shtml), and identification of the retrieved other mosquito oxidoreductin-like proteins were confirmed by blasting against the VectorBase (http://cpipiens.vectorbase.org/Tools/BLAST/).

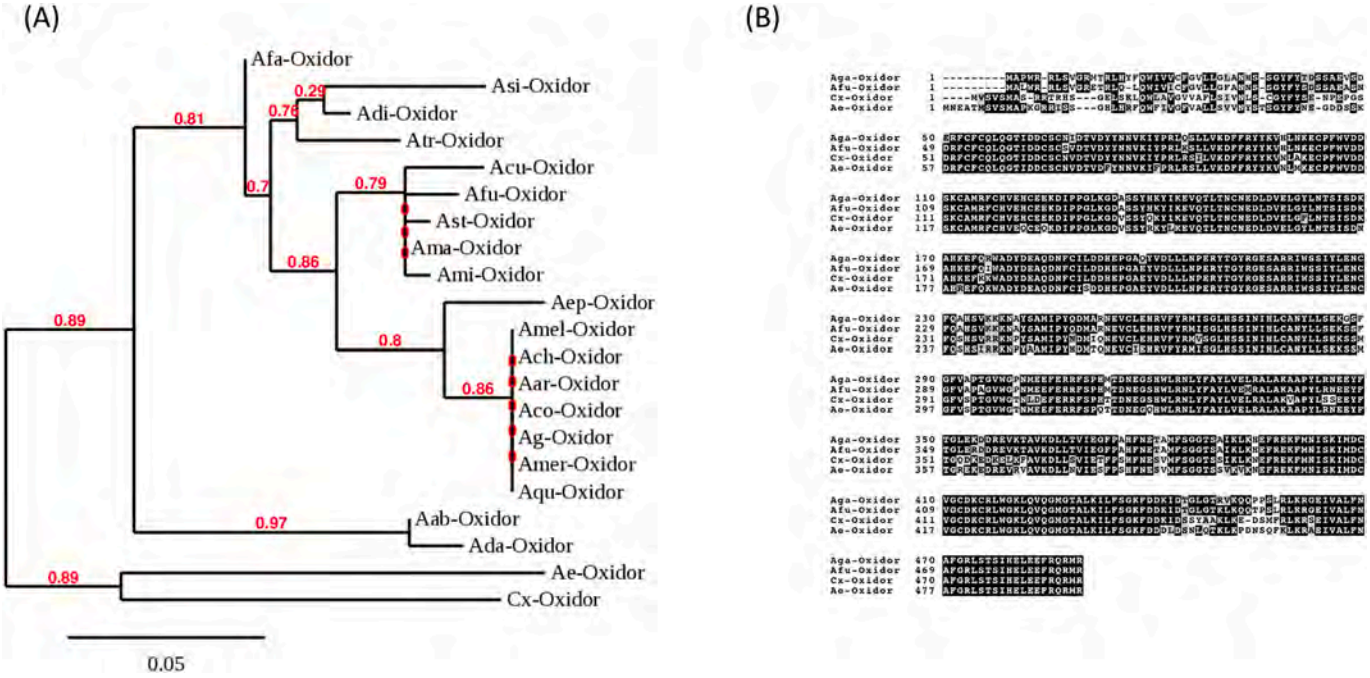

Fig. 1. (A) Phylogenetic tree of mosquito oxidoreductase-like-protein (Oxidor) generated with the maximum likelihood method and bootstrap analysis. Bootstrap values are shown at the base of the branches and represent the percentage of times that grouping was supported. Cx-Oxidor, *Culex pipiens*, CPIJ004301. Ae-Oxidor, *Aedes aegypti*, AAEL002102. Ada-Oxidor, *Anopheles darlingi*, ADAC006477. Aab-Oxidor, *Anopheles albimanus*, AALB008901. Aep-Oxidor, *Anopheles epiroticus*, AEPI001005. Amel-Oxidor, *Anopheles melas*, AMEC006245. Ach-Oxidor, *Anopheles christyi*, ACHR008565. Aar-Oxidor, *Anopheles arabiensis*, AARA004638. Aco-Oxidor, *Anopheles coluzzii*, ACOM037629. Ag-Oxidor, *Anopheles gambiae*, AGAP002816. Amer-Oxidor, *Anopheles merus*, AMEM002911. Aqu-Oxidor, *Anopheles quadriannulatus*, AQUA009382. Acu-Oxidor, *Anopheles culicifacies*, ACUA005265. Afu-Oxidor, *Anopheles funestus*, AFUN007955. Ast-Oxidor, *Anopheles stephensi*, ASTE002083. Ama-Oxidor, *Anopheles maculatus*, AMAM018691. Ami-Oxidor, *Anopheles minimus*, AMIN003658. Afa-Oxidor, *Anopheles farauti*, AFAF012442. Atr-Oxidor, *Anopheles atroparvus*, AATE006712. Adi-Oxidor, *Anopheles dirus*, ADIR003499. Asi-Oxidor, *Anopheles sinensis*, ASIS004002. (B) Alignment of the oxidoreductase-like proteins from *Culex pipiens* (Cx-Oxidor), *An. gambiae* (Aga-Oxidor), *Ae. aegypti* (Ae-Oxidor) and *An. funestus* (Afu-Oxidor). A potential oxidoreductase domain is located alignment region 79–480 a.a.. The black and gray shades indicate similar amino acids, respectively.

The evolutionary history was inferred using the maximum likelihood (ML) method (Guindon and Olivier Gascuel, 2003; Guindon et al., 2005). The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the mosquito oxidoreductin-like proteins analyzed (Fig. 1). The percentage of replicate trees in which the associated oxidoreductin-like proteins clustered together in the bootstrap test (500 replicates) is shown next 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.

The deduced amino acid sequence of *Culex oxidor* gene was assembled, analyzed, and aligned with a.a sequences of *oxidor* homologues of the mosquitoes *Anopheles gambiae*, *Anopheles funestus* and *Aedes aegypti and* (accession numbers AGAP002816 for *oxidor* of *An. gambiae*, AFUN007955 for *An. funestus*, AAEL012847 for *Ae. aegypti*). Multiple sequence alignment of the four a.a sequences was performed using the CLUSTALW2 program (https://www.ebi.ac.uk/Tools/msa/muscle/). Protein domains were identified by searching the pfam database (http://pfam.xfam.org).

2.3. Synthetic dicer-substrate siRNA injection into adult female mosquitos

Targeting genes that encoding oxidoreductin-like protein (*oxidor*, CPIJ004301) was performed as previously described (Sim and Denlinger, 2008). Dsi-*oxidor* corresponds to target sequences on exon 1. The sequences of chosen siRNA duplexes are confirmed through BLAST searches and have no significant homology to *Cx. pipiens* genes other than for *oxidor,* which are as follows: dsi-*oxidor*: 5′-rCrArUrGrArArUrArCrUrUrGrUrUrGrArUrArGrUrCrCrUrUrCrCrA-3′/ 5′-rGrGrArArGrArCrUrArUrCrArArCr ArArGrUrArUrUrCrATG-3′. A scrambled negative control dsiRNA, an dsi-control duplexes lacking significant sequence homology to any genes in the *Cx. pipiens* genome, is used for control experiments, dsi-control: 5′rGrArArGrArGrCrArCr UrGrArUrArGrArUrGrUrUrArGrCGT-3′/ 5′rArCrGrCrUrArArCrArUrCrUrArUrCrArGr UrGrCrUrCrUrUrCrCrG-3′. None of the phenotypes reported are observed in control-injected females, which are not significantly different than wild type females for any of the phenotypes assessed.

2.4. RNAi efficiency evaluation using qRT-PCR

We carried out qRT-PCR of the dsi-RNA-injected mosquitoes as previously described (King et al., 2019). Briefly, total RNA samples were extracted with TRIzol (Invitrogen) from three batches of 15 adult female mosquitoes on various days after dsi-RNA injection. To remove genomic DNA contamination, RNA samples were treated with DNase I following the manufacturer's instructions (50–375 units/μl; Invitrogen). For reverse transcription, 5 μg of total RNA was reverse-transcribed with SuperScript III RNase H-reverse transcriptase (Invitrogen). All reactions were performed in triplicate in a total volume of 20 μl containing 10 μl SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA) and 300 nmol of each primer at the following conditions: 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The following qRT-PCR primers were used: q-oxidor, CATTCGCAGCTGAACTACAA and TGGCTACGTGCCAAAATAGA; q-rpl19, CGCTTTGTTTGATCGTGTGT and CCAATCCAGGAGTGCTTTTG. The ribosomal protein large subunit 19 gene (rpl19) was used as a loading control. Expression data were normalized to the geometric mean of housekeeping gene rpl19 to control the variability in expression levels and were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Then, the statistical significance of differences in transcript levels were determined using a Student's *t*-test between the relative transcript values of dsi-oxidor injected vs. control samples (dsi-control injected), using three biologically independent replicates for each gene. A *P*-value less than 0.05 was considered as a significant transcript-level change.

2.5. Follicle degeneration following Dsi-oxidor injection

Within a day after eclosion, diapause-destined females were injected in the thorax with dsi-*oxidor* or dsi-*control*. Each treated cohort was kept in a 24 cm × 24 cm × 24 cm cage. A week later, ovaries were dissected in mosquito saline and dipped in Acridine Orange (Cayman Chemical Company, Ann Arbor, MI; 50 μmol/ml in mosquito saline) and Neutral Red (Sigma N2880-25G) at pH 6 for 1 min to assist visualization of the follicles, and they are then immediately examined using fluorescent filters. The number of follicles per female containing condensed nuclei was recorded, and if more than 5 follicles per female exhibit condensed nuclei as shown by staining with Acridine Orange, the female is considered to be in the process of follicle degeneration as described previously (Sim and Denlinger, 2011).

2.6. Survival analysis

To evaluate the knockdown effect of *oxidor* on the survival rate, a total of 135 mosquitoes per cohort were intrathoracically injected with ~0.5 μl of dsi-*oxidor* or dsi-*control*. Mosquitoes were held at 18 °C, 75% relative humidity, and 9:15 L:D cycle, with access to sugar, and survival is assessed every five days. Survival curves were fitted and analyzed using Cox proportional hazards model (R version 4.0.4, "survival", "survminer" packages) (R Core Team, 2014). Univariate Cox regression and Wald test were used for testing the statistical significance of the differences between the dsi-oxidor and dsi-control injected mosquitoes (Cox, 1972). Individuals who did not die by 35 d after dsi-*control* injections were censored (0 = death event did not occur; 1 = death event occurred). The individual females with dsi-*oxidor* injections were not censored, because the experiment finished with the death of all dsi-oxidor injected females.

3. Results

3.1. Phylogenetic analysis of Culex pipiens oxidor

We identified other mosquito *oxidors* from *Cx. pipiens* by performing BLAST searches on the non-redundant genomic database using sequences of *Cx. pipiens* oxidoreductin-like genes. The sequences of *Cx. pipiens oxidor* shared the highest identities, 85% with *oxidor* from a closely related mosquito, *Aedes albopictus*. The 1470-bp mRNA fragment of *Cx. pipiens oxidor* shared 84% identity with *oxidor* from *Aedes aegypti* and 82% identity to *oxidor* sequences from *Anopheles gambiae*. The deduced amino acid sequences *oxidor*, based on a pfam search, belong to an oxidoreductin-1 family (Ero1) which is an essential oxidoreductase that oxidizes proteins in the endoplasmic reticulum to produce disulfide bonds (Frand et al., 2000). The activity of Ero1 is regulated by Pdi1, which is a protein disulfide isomerase. This regulation of Ero1 through reduction and oxidation of regulatory bonds within Ero1 is essential for maintaining the proper balance in the ER (Kim et al., 2012; Baker et al., 2008). A phylogenetic analysis of known mosquito oxidors was performed to infer relationships of the *Culex* oxidor with those identified in *Ae. aegypti* and *An. gambiae* mosquitoes (Fig. 1). Oxidor is fairly conserved between *Cx. pipiens* and *Ae. aegypti*, but based on phylogenetic analysis, *Anopheles* mosquitoes are evolutionarily more distant, a result which is consistent with reported speciation patterns within the family Culicidae (Krzywinski et al., 2006).

3.2. Transcript levels of oxidor from diapausing and nondiapausing females of Cx. pipiens and RNAi efficiency evaluation using qRT-PCR

The transcript levels of the *oxidor* gene in nondiapausing and diapausing *Cx. pipiens* were obtained using qRT-PCR. The expression patterns of *oxidor* showed that it was up-regulated in diapausing mosquitoes one week after eclosion but were down-regulated in nondiapausing mosquitoes reared at long-day (LD) conditions (Fig. 2A).

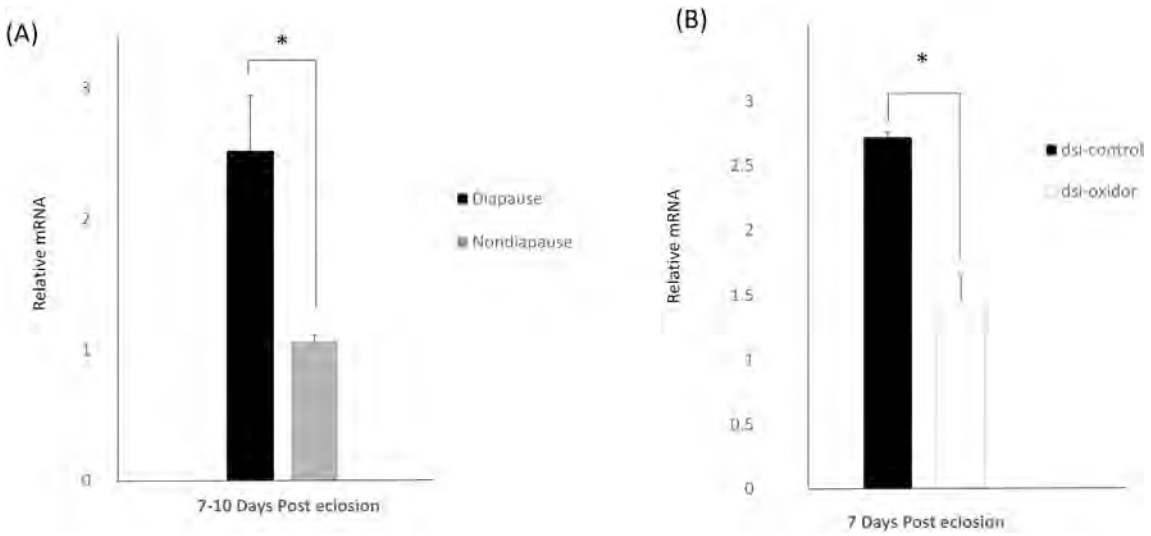

Fig. 2. (A) Quantitative Real-Time PCR showing expression of the gene encoding oxidoreductase-like protein (oxidor) in diapausing (D) and nondiapausing (ND) *Cx. pipiens* females 7–10 d post eclosion (dpe). ND (gray bar) = programmed by long daylength for nondiapause, D (black bar) = programmed by short daylength for diapause. Both groups were maintained at 18 °C. (B) RNA interference efficiency targeting oxidoreductin-like protein (dsi-Oxidor) in *Cx. pipiens*. Transcript levels of the gene encoding oxidoreductin-like protein in females injected with dsi-oxidor (white bar) were compared with the dsi-control (black bars). Expression levels were measured by qRT-PCR at 7 days after dsi-RNA injection. qRT-PCR data were normalized using a ribosomal protein large subunit 19 (rpl19) as a loading control. Bars (mean ± s.e., $n = 3$ groups of 10 individuals each) with asterisks (*) indicate significant differences at $P < 0.05$, *t*-test.

In non-diapausing females, *oxidor* levels are relatively low in comparison to diapausing females. Increased expression of the *oxidor* gene in mosquitoes in response to short daylength (destined to diapause) suggested the possibility that this gene may be involved in regulating oxidative stress during early diapause period.

In addition, RNAi efficiency was assessed by qRT-PCR. In contrast to the relatively high induction of *oxidor* in dsi-*control* injected mosquitoes, more than 50% reduction of *oxidor* transcript was observed in dsi-*oxidor* injected, diapausing mosquitoes, using qRT−PCR and primers corresponding to the *oxidor* gene (Fig. 2B). This result shows that the injection of dsi-*oxidor* successfully inhibited the activity of the *oxidor* gene in diapausing females. It was also shown that the level of suppressed

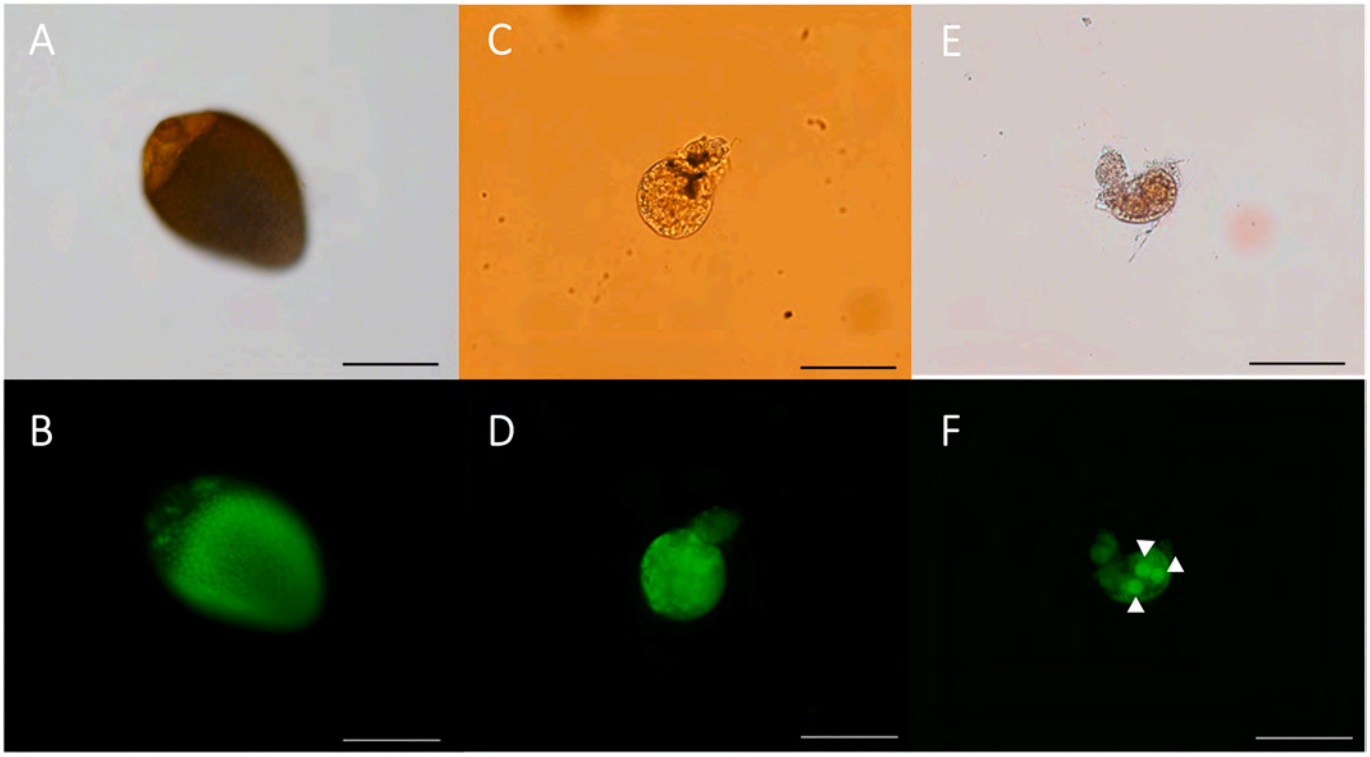

Fig. 3. Primary follicles from ND and D females, prepared with neutral red and acridine orange post adult eclosion under white light (top) and GFP filter (bottom). (A, B) Primary follicles of ND females 7–10 dpe and with dsi-control injection. (C, D) Primary follicles of D females 7–10 dpe and with dsi-control injection. (E, F) Primary follicles of D females 7–10 dpe and with dsi-oxidor injection. The triangles indicate the condensed nuclei which are in the process of apoptosis. Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression of this gene was similar to that of the non-diapausing counterparts. Ribosomal protein L19 (RpL19), the loading control used to normalize transcript levels, showed no difference in expression 7 days post-injection (Fig. 2B), thus indicating that the low expression levels observed for the *oxidor* gene were related to the knockdown effect of dsi-*oxidor* injections rather than variation in sample loading.

3.3. Dicer-substrate RNAi-oxidor (dsi-oxidor) increases degeneration of follicles in diapausing females

Three days after injection of dsi-*oxidor* or dsi-*control*, no significant differences were observed in the ovarian follicle degeneration in both treatments. However, after 7 days post dsi-RNAs injection, ovaries were examined again, and degeneration rate of the follicles was signifcantly higher in dsi-*oxidor* treated females compared to those of control cohorts. Degenerating follicles, which were detected by staining with Neutral Red, were observed in dsi-*oxidor* injected mosquitoes, and Acridine Orange staining of the same ovaries revealed condensed nuclear chromatin (Fig. 3 and Table 1). One of the important characteristics of mosquito wintering diapause is a mechanism that effectively suppresses the damage caused by reactive oxygen species in follicle cells where the normal development process is halted. The experimental results that inhibit the function of the *oxidor* gene suggest that this gene product effectively protects against follicle cell damage caused by a reactive oxygen species. (See Fig. 4.)

3.4. Reduced survival of diapausing Cx. pipiens in response to dsi-oxidor injection

Survival analysis using the Cox's Proportional Hazards model showed a higher death risk (hazard ratio; HR) for dsi-oxidor injected females. Hazard ratio estimate of exp.(coef) = 19.71 with 95% CI (11.98, 32.44). This result suggests that, at least with early diapause, the reduction of expression of *oxidor* significantly decreases mortality of diapausing *Cx. pipiens,* due to possible consequences of reduced antioxidant activity.

4. Discussion

In *Cx. pipiens, oxidor* (oxidoreductin-like protein) is homologous to oxidoreductin-like protein ERO1 in other organisms. Oxidoreductin-like proteins are found in the endoplasmic reticulum and interact with protein disulfide isomerases (PDI) for protein folding. Oxidoreductin-like proteins reoxidize and in turn reactivate PDI for new rounds of oxidative protein folding (Zito, 2015). ERO1 activate this process by transferring electrons from molecular oxygen with the use of ERO1's cofactor FAD1, and in turn creates H_2O_2 (Tu and Weissman, 2002). In yeast, ERO1-1 mutants have been shown to be non-viable when grown in non-permissive temperatures, and slower growth rates at permissive temperatures compared to wild type yeasts (Araki and Nagata, 2011; Frand and Kaiser, 1998). In the presence of DTT to stimulate reducing effects in the cell, ERO1 knockouts are completely unviable (Pollard et al., 1998), and another study shows that double mutants ERO1 and FAD1 have completely unviable cell cultures as well (Tu and Weissman, 2002). Oxidative stress responses such as catalase, detoxify H_2O_2 into H_2O and O_2 (Sim and Denlinger, 2011), and with the formation of O_2, gets recycled to be used by ERO1 for protein folding. In *Bombyx mori*, expression of bEro1 mRNA was markedly increased during endoplasmic reticulum (ER) stress by H_2O_2, Monecin, dithiothreitol and tunicamycin (Seo et al., 2015), which coincides with increases in oxidative stress. In *Saccharomyces cerevisiae*, disulfide bonds are formed by thiol-disulfide transfer mechanisms with the oxidized Pdi1p (Kim et al., 2012). ERO1 is important for protein folding so signaling pathways to occur, and if protein folding does not occur, mutations and eventual cell death can occur from an extended unfolded protein response (Haynes et al., 2004).

In dauer stage *Caenorhabditis elegans*, notch receptor LIN-12 cooperates with the insulin signaling pathway to signal recovery from dauer stage, and if LIN-12 is compromised, dauer stage *C. elegans* are unable to recover out of this phenotype (Ouellet et al., 2008). In Drosophila, Notch protein is important for cell signaling, which is folded in the ER by ERO1, and if folding does not occur, defects in lateral inhibition and inductive signaling occur (Tien et al., 2008). Notch is also important for many signaling pathways including tissue organization and chromatin organization, and defects can occur if Notch is not folded properly to carry out cellular communication (Bray, 2016). In *Aedes* mosquitoes, when AaNotch is knocked down with RNAi, fertility and fecundity are drastically decreased, and egg vitality is decreased drastically as well.

In diapausing insects, the ability to efficiently remove oxidative stress for overwintering survival is crucial (Zhao and Shi, 2010). During our investigation, the oxidoreductin-like protein, *oxidor*, was shown to be upregulated one week after eclosion of adult diapausing females compared to non-diapausing counterparts. When *oxidor* was knocked down in diapausing females, mortalitiy increased significantly, which was observed when the genes encoding FOXO, Catalase, Superoxide dismutase-2 and Glycogen synthase (Sim and Denlinger, 2008, 2011; King et al., 2019) were knocked down. These results thus point that the oxidoreductin-like protein is related to these genes in respect to detoxify reactive oxygen species in diapausing female mosquitoes at least in early-phase of overwintering diapause.

Oxidor is an antioxidant-related gene that was previously identified during ChIP-seq analysis as a potential for reducing oxidative stress during mosquito diapause (Sim et al., 2015). This gene was shown to be highly upregulated in diapausing female mosquitoes one week after adult eclosion in comparison to control populations. When *oxidor* was suppressed using dsi-RNAs, the proportion of degenerating follicles increased substantially in diapausing females compared to nondiapausing counterparts. Follicles from diapausing females that had *oxidor* suppressed using dsi-RNAs, an increase in condensed chromatin was

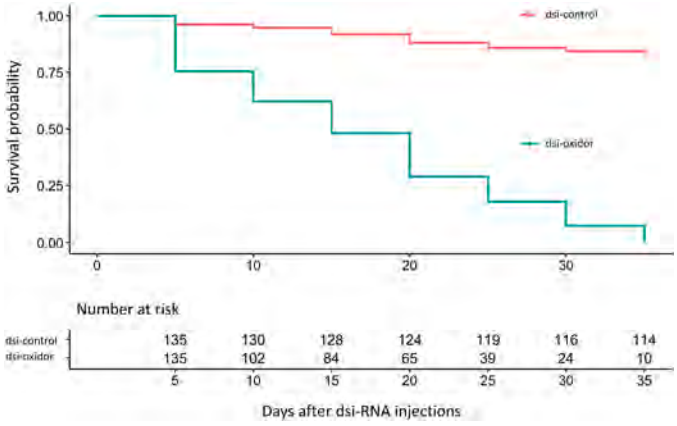

Fig. 4. The survival probability of female mosquitoes after injection of dsi-RNAs targeting against the gene encoding oxidoreductin-like protein (dsi-oxidor) and control (dsi-control). Survival curves were fitted and analyzed using Cox proportional hazards model. $N = 135$ individuals per each group.

Table 1
Proportion of diapausing *Cx. pipiens* with ovarian follicles in a state of degeneration following an injection of dsi-Oxidor.

Programmed for diapause	N	Females with degenerating follicles (%)
Dsi-control day 3	36	13.29
Dsi-oxidor day 3	36	19.17
Dsi-control day 7	36	15.62
Dsi-oxidor day 7	36	62.38*

* Indicates significant differences from untreated controls (X^2- goodness of fit test at $P < 0.05$ and d.f. = 1).

observed after staining with Acridine orange, which is a key characteristic of cellular apoptosis. In addition, the survival of diapausing females drastically decreased at 7–10 days after the injection of dsi-RNAs targeting the *oxidor* gene, and continued a downward trend of survival while diapausing mosquitoes that were injected with the control dsi-RNAs showed no significant decrease in survivability even after 30 d after the injections. This result is similar organismal response when the activity of the *FOXO* gene was suppressed (Sim and Denlinger, 2008). It may support the fact that *oxidor* is one of the downstream genes under the control of FOXO transcriptional factor, which is activated at the onset of diapause program in the mosquito *Cx. pipiens*.

It has been shown in previous research that the insulin/FOXO pathway is a key molecular switch for initiating and maintaining overwintering diapause in the mosquito *Cx. pipiens* (Sim and Denlinger, 2008), and ChIP-seq analysis using FOXO antibody found the potential target genes involved in this diapause program. Among them, several genes are linked to overwintering stress tolerance for reducing oxidative stress based on the gene ontology analysis (Sim et al., 2015; Sim and Denlinger, 2011). The insulin/FOXO signaling pathway is also highly conserved in other model organisms such as the fruit fly *D. melanogaster* and the nematode, *C. elegans*, and similar phenotypes were elucidated including oxidative stress tolerance in these organisms. For example, fly FOXO activation increases stress tolerance through SODs (Honda and Honda, 1999; Mattila et al., 2009), and when dFOXO is knocked down, sensitivity to oxidative stress increases in the fly *D. melanogaster* (Jünger et al., 2003).

In conclusion, we have demonstrated that the gene encoding an oxidoreductin-like protein offers a role in protecting diapausing mosquitoes and also plays a key role in protecting diapausing ovaries from potential oxidative damage. However, additional research is needed on how this gene plays a role in oxidation-related processes other than diapause, as well as in other forms of diapause in different insects.

Author contribution

C.S. and B.K. designed research; B.K., A.I. and M.L. performed research and analyzed data; and C.S., and B.K. wrote the paper.

Data deposition

The sequences reported in this paper have been deposited in the Genbank database (accession no. *Culex pipiens oxidor*, KP057868).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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