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Catalase and superoxide dismutase-2 enhance survival and protect ovaries during overwintering diapause in the mosquito *Culex pipiens*

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Abstract

Lifespan extension and stress resistance are two important features of diapause that are essential for successful overwintering. We present several lines of evidence suggesting that genes encoding two antioxidant enzymes, *catalase* and *superoxide dismutase-2*, are critical in generating these characteristics during diapause in overwintering adults of the mosquito *Culex pipiens*. Expression of both *catalase* and *sod-2* was dramatically higher in young diapausing females than in their nondiapausing counterparts at the same age. Suppression of *catalase*, but not *sod-2*, resulted in increased damage to the ovaries, as evidenced by signs of apoptosis in ovarian follicle cells. Adult survival time was shortened when levels of either *catalase* or *sod-2* were suppressed using RNAi. Together these results imply that these two antioxidants are particularly important in promoting survival in diapausing females, while elevation of *catalase* also contributes to protection of the ovaries. In addition, RNAi directed against forkhead transcription factor (*foxo*), a gene thought to be upstream of the genes encoding these antioxidants, resulted in suppression of both *catalase* and *sod-2*. The linkage with FOXO suggests that the genes encoding these two antioxidants are components of an important gene network regulated by this transcription factor.

1. Introduction

Lifespan in animals is a dynamically controlled process dependent upon integration of diverse environmental, humoral and genetic factors. In the nematode *Caenorhabditis elegans* and the fly *Drosophila melanogaster* genetic screens identified insulin/forkhead transcription factor as a key signaling pathway regulating lifespan. Interestingly, many of the genes found in the screening for extended lifespan are the same as those identified in studies of dauer formation (a diapause-like state) in the nematode and fly diapause (Lee et al., 2001; Murphy et al., 2003; Tatar et al., 2001; Williams et al., 2006). In many ways the essence of diapause is a huge extension of the lifespan (Denlinger, 2008). The diapause syndrome may thus offer an interesting state for probing genes involved in lifespan

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extension. Recently, completion of numerous genome projects covering diverse organisms adds a genomics perspective to aging and diapause research. New genome information available for the mosquito *Culex quinquefasciatus* (Arensburger et al., 2010), a sister species of *C. pipiens*, offers a valuable tool for probing diapause in *C. pipiens*, a species with a distinctive adult diapause that has been well documented (Barr, 1957; Mitchell, 1983; Mitchell and Briegel, 1989a, b; Sanburg and Larsen, 1973; Spielman, 1964, 1971). This mosquito is an important vector of West Nile virus in North America, and understanding its diapause may suggest new tools for disrupting the vulnerable overwintering population.

Recent work on the molecular regulation of diapause in *C. pipiens* points to the importance of insulin and forkhead transcription factor (FOXO) as key elements in the signaling pathway leading to the diapause phenotype. Briefly, the insulin signaling pathway is not activated in diapausing adult females, thus lifting suppression of FOXO. This, in turn, is thought to lead to induction of target genes associated with the diapause syndrome, including enhanced stress tolerance, extended lifespan, and accumulation of fat reserves (Sim and Denlinger, 2008, 2009a, b). This mechanism is well conserved, as evidenced from results with the nematode *C. elegans* and the fly *D. melanogaster* (Giannakou and Partridge, 2007; Lee et al., 2001). Previous transcriptome-wide studies of these two organisms have shown that up-regulation of *foxo* increases lifespan and is associated with the up-regulation of genes involved in cold tolerance, UV resistance, and resistance to reactive oxygen species (ROS) (Gershman et al., 2007; McElwee et al., 2003; Murphy et al., 2003; Oh et al., 2006). The involvement of protective agents during diapause was also observed in *C. pipiens*: heat shock proteins and actins are up-regulated in diapausing females to enhance cold tolerance (Kim et al., 2006; Rinehart et al., 2006; Robich et al., 2007). In addition, when we injected the antioxidant enzyme mimic Mn(III)TBAP into mosquitoes after a systemic suppression of *foxo* by RNAi, mosquito survival was enhanced (Sim and Denlinger, 2008). These results thus suggest that diapausing mosquitoes efficiently counter the oxidative damage that accumulates during overwintering, possibly contributing to the 5-fold increase in the lifespan of a diapausing versus nondiapausing female, a feature that is essential if the female is to survive until the following spring. Two complementary scenarios could be envisioned: the diapausing mosquito could either decrease ROS production or activate its ROS detoxification system as a mechanism to combat oxidative stress.

Several theories have been proposed as mechanisms for implementing an extended lifespan. A ROS detoxification mechanism is one of the well-known mechanisms for maintaining homeostasis and increasing lifespan. The ROS include superoxide ion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and peroxy radical ($OH^{\bullet}$), which can be generated as a byproduct through activation of NADPH oxidase, nitric oxide synthase, and mitochondrial electron transfer (Bashan et al., 2009). Oxidative stress occurs when there is an imbalance between oxidant generation and antioxidant defense (Bashan et al., 2009). Thus, the antioxidant defense mechanism relies on enzymes that catalyze ROS synthesis and regeneration. The three major ROS detoxifying enzymes include superoxide dismutase, which converts superoxide ion ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2), catalase and glutathione peroxidases (Gpx) which detoxify hydrogen peroxide (H_2O_2).

In this study, we propose that these antioxidant enzymes contribute significantly to the extended lifespan and enhanced stress tolerance of the diapausing mosquito *C. pipiens*. Five genes encoding ROS detoxification enzymes, three superoxide dismutases (SOD-1, -2 and -3), catalase, and glutathione peroxidase, were cloned from the mosquito *C. pipiens*. Expression levels of these genes were monitored in both diapausing and nondiapausing mosquitoes during the first 10 days after adult eclosion, and RNA interference was then used to evaluate function.

2. Material & Methods

2.1 Insect Rearing

The stock colony of *C. pipiens* (Buckeye Strain) was reared at 25°C and 75% relative humidity under a 15 h light: 9 h dark (L:D) photoperiod, as previously described (Robich and Denlinger, 2005). When larvae reached the second instar, rearing containers were placed under one of two environmental conditions: nondiapausing (ND) adults were generated by rearing at 18°C, 75% RH, and 15:9 L:D. To induce diapause (D), mosquitoes were reared at 18°C, 75% RH, and 9:15 L:D. To confirm diapause status, primary follicle and germarium lengths were measured, and the stage of ovarian development was determined according to described methods (Christophers, 1911).

2.2 Identification and bioinformatic analysis of *Culex* superoxide dismutase, catalase, glutathione peroxidase sequences

To retrieve sequences of *Culex* superoxide dismutase (SOD), glutathione peroxidase (Gpx), catalase sequences, *Anopheles* oxidoreductase genes were utilized in BLASTN searches on genome data from Vectorbase (<http://cpipiens.vectorbase.org/Tools/BLAST>). RT-PCR was used to clone the putative genes in *C. pipiens*. Total RNA samples were extracted using Trizol (Invitrogen, CA). To remove genomic DNA contamination, RNA samples were treated with 1.0 µl DNase I following the manufacturer's instructions (50–375 units/µl; Invitrogen, CA). For reverse transcription, 5 µg total RNA was reverse transcribed with Superscript III RNase H-reverse transcriptase (Invitrogen). cDNAs containing oxidoreductase coding regions were amplified using the following primers (5'–3'): *sod-1*, GAAGAACGACGTCTCGAAGG and CCCGATCGCTGCTAGTACA; *sod-2*, AGCACAGCTGGAGTGGTAGG and CCTCCATAAA GCGTCAAAA; *sod-3*, GCTTCCACATCCACGAGTTT and TTTGAAATGACCAAACAA CGA; *catalase*, TATGGATCGCACACCTTCAA and TCTTGATCGACACGCGATAC; *gpx*, CGAAGTCAGCCTCAGCAAGT and CCTCCAGCAAATAAGGATGC and identities of the retrieved sequences were confirmed by performing BLASTN searches against Vectorbase (<http://www.vectorbase.org/Tools/BLAST>). Protein domains were identified by searching the pfam database (<http://pfam.sanger.ac.uk>).

2.3 Transcript levels in diapause and nondiapause *C. pipiens* using qRT-PCR

To compare transcript levels of *sods*, *gpx* and *catalase* in diapausing and nondiapausing mosquitoes, total RNA samples were extracted with Trizol (Invitrogen, CA) from three batches of 15 females 7–10 days after adult eclosion. Genomic DNA contamination was removed, and reverse transcription was performed as described above. qRT-PCR was performed using an iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA), as described previously (Sim and Denlinger, 2009b). To select appropriate qRT-PCR primer sets, primers were used only if the amplification efficiency was > 0.99 and < 1.01 and only if the dissociation curve of each primer set yielded a single peak. Standard curves were generated for each transcript tested using 10-fold serial dilutions of plasmids that included fragments of *sods*, *gpx* and *catalase* genes, ranging from 100 to 0.01 pg per reaction. These curves, based on linear regression, were generated with plasmid input transformed by natural logarithm as the dependent variable and Ct numbers as the independent variable. Ct numbers from cDNA samples were then used in the linear regressions to estimate transcript levels for each gene.

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 30s. The following qRT-PCR

primer sets were used: qSOD-1, AGAAGCACCAACGCCTAC and CCTTCGAGACGTCGTTCTTC; qSOD-2, GCATTGCGAAAACCTTCCTTC and TGCCAGATCATCAATTTCA; qSOD-3, GAGCAAG ACCACCGGAAAT and GATTCGTTGCGTCAACACTG; qCatalase, CAAGTGATGACCTT CGAGCA and TTTTACCGACTGGGATCAGC; qGpx, CCTAGCGTCACTGCACAAAA and TTTTCGTTGCGAGATTCAGT. The *ribosomal protein large subunit 19* gene (*rpl19*) was used as a loading control. Geometric averages of transcript levels of the internal controls were used to normalize transcript levels of each gene. Then, statistical significance of differences in transcript levels were determined using a Student's t-test between the relative transcript values of D vs ND samples, using three biologically independent replicates for each gene. A P-value less than 0.05 was considered to be a significant transcript level change.

2.4 dsRNA preparation and injection into adult female mosquitoes

dsRNA for the *C. pipiens* *sod-2* and *catalase* genes were prepared using the MEGAscript T7 transcription kit (Ambion, Austin, TX), as described (Sim and Denlinger, 2008). The PCR products for in vitro transcription were amplified using the following primer sets: dsCatalase, TAATACGACTC ACTATAGGGTATGGATCGCACACCTTCAA and TAATACGACTCACTATAGGGTCTTGAT CGACACGCGATAC; dsSOD-2, TAATACGACTCACTATAGGGAGCACAGCTGGAGTGGT AGG and TAATACGACT CACTATAGGGCCTCCATAAAGGCGTCAAAA. Each PCR-derived fragment was sequenced and blasted against the *C. quinquefasciatus* genome database (<http://cpipiens.vectorbase.org/Tools/BLAST/>) to validate redundancy of the sequence and to confirm unique sequences. In knockdown experiment with D mosquitoes, ~ 0.7 µl dsRNA of the *Culex* *sod-2* and *catalase* (1.5 µg/µl) were injected into the thorax of cold-anesthetized females using a microinjector (Tritech Research, Los Angeles, CA). ~ 0.7 µl dsRNA of β -galactosidase and *foxo* (1.5 µg/µl) were also used for dsRNA injection experiment as described (Sim and Denlinger, 2008).

2.5 RNAi efficiency evaluation using qRT-PCR

qRT-PCR of the dsRNA injected mosquitoes was carried out as above and as described (Sim and Denlinger, 2009b). To evaluate RNAi efficiency, primers were used to amplify endogenous *foxo*, *sod-2* and *catalase*. The *rpl19* from *C. pipiens* was used as a loading control.

2.6 Follicle degeneration following dsSOD-2 and dsCatalase

Within a day after eclosion, D females were injected in the thorax with dsSOD-2, dsCatalase or ds β -gal (control). Each treated cohort was kept in an 8 cm diameter $\times$ 12 cm cage. Cotton soaked in 10 % sucrose was provided 1 h after the dsRNA injection. Cages were placed at 18 °C, 75 % R.H., 9: 15 h L:D, and ovaries were assessed one week after injection. All groups were chilled 2–3 min at –20 °C prior to dissection. Ovaries were dissected in mosquito saline (150 mmol/l NaCl, 4mmol/l KCl, 1mmol/l CaCl₂, 0.1 mmol/l NaHCO₃, 0.6 mmol/l MgCl₂ buffered with 25 mmol/l Hepes) and dipped in Neutral Red (Sigma N2880-25G) at pH 6 for 1 min to assist visualization of the follicles (Bell and Bohm, 1975). Neutral Red concentrates in lysosomes and yields a deep red color at a slightly acid pH (Hopwood et al., 2001; Wilson, 1990). Degenerating follicles exhibit increased uptake of Neutral Red (Bell and Bohm, 1975; Clements and Boocock, 1984). The number of degenerating follicles was recorded. Acridine Orange (50 µmol/l in mosquito saline) was then added to the ovaries, and they were immediately examined using fluorescent filters. The number of follicles/female containing condensed nuclei was recorded, and if more than 5 follicles/female exhibited condensed nuclei as noted by staining with Acridine Orange, the female was considered to be in the process of follicle degeneration.

2.7 Survival following injection of dsSOD-2 and dsCatalase

To evaluate the knockdown effect of *sod-2* and *catalase* on survival of D mosquitoes, 20 females per cohort were intrathoracically injected with either ~0.7 µl dsSOD-2, dsCatalase, or dsβ-gal (1.5 µg/µl). Mosquitoes were held at 18 °C, 75% R.H. and a 9:15 L:D cycle, with access to sugar, and survival was assessed on days 1, 5 and 10. Experiments were replicated four times.

3. Results

3.1 *C. pipiens* SODs, Catalase and Glutathione Peroxidase

The 510-bp cDNA fragment of *C. pipiens catalase* shared 81% identity with *catalase* (AAEL013407) from the mosquito, *Aedes aegypti* (supplementary information (SI) Fig. S1). The deduced amino acid catalase sequence, based on a pfam search, belongs to a family of catalases (PF00199) which are antioxidant enzymes that catalyze the conversion of hydrogen peroxide to water and molecular oxygen, serving to protect cells from its toxic effects (Bashan et al., 2009).

The 538-bp cDNA fragment of *C. pipiens superoxide dismutase-1* (SOD-1) shared highest identity (83%) with SOD-1 (AAEL004823) from a closely related mosquito, *Ae. aegypti*; the 429-bp cDNA fragment of *C. pipiens* SOD-2 shared highest identity (79%) with *Anopheles gambiae* SOD-2 (AGAP005234); the 324-bp cDNA fragment of *C. pipiens* SOD-3 shared highest identity (82%) with *Ae. aegypti* SOD-3 (AAEL011498) (Fig. S2). The deduced amino acid SOD sequences, based on a pfam search, belong to a family of superoxide dismutases (PF00081) which catalyze the conversion of superoxide radicals to molecular oxygen. Their function is to destroy radicals that are normally produced within cells and are toxic to biological systems (Bashan et al., 2009).

The 408-bp cDNA fragment of *C. pipiens glutathione peroxidase* (Gpx) shared 96 % identity with Gpx (AAEL000495) from the mosquito, *Ae. aegypti* (Fig. S3). The deduced amino acid glutathione peroxidase sequence, based on a pfam search, belongs to a family of glutathione peroxidases (PF00255) that catalyze the reduction of hydroperoxides by glutathione. Its main function is to protect against the damaging effect of endogenously formed hydroperoxides (Bashan et al., 2009). Vectorbase identities of these five oxidoreductase sequences from *C. pipiens* are listed in captions for Supplemental Figures 1–3.

3.2 Transcript levels of catalase, sod-1, -2, -3, and gpx in diapausing vs. nondiapausing *C. pipiens*

Expression patterns of *catalase*, *sod-1*, -2, -3, and *gpx* in diapausing and nondiapausing *C. pipiens* were obtained using qRT-PCR. The mRNA expression patterns of *catalase* and *sod-2* indicated much higher expression levels in diapausing than in nondiapausing females, both at 3 days and one week after eclosion (Fig. 1). In contrast, mRNA expression levels of *sod-1*, -3 and *gpx* were similar in the two types of mosquitoes (Fig. 1). Up-regulation of the *catalase* and *sod-2* genes in mosquitoes in response to short daylength (destined to diapause) may reflect increased antioxidant capacity and may in fact represent a biological response to increased oxidative stress, at least in early diapause.

3.3 Transcript level of *foxo* is correlated with expression of catalase and sod-2 in diapausing females

RNAi targeting *foxo* resulted in a dramatic decrease of *foxo* expression in diapausing females (Fig. 2). Interestingly, suppression of *foxo* also resulted in suppression of both *catalase* and *sod-2*. The knock-down was nearly total on day 1. By day 3 the decrease in

expression was still approximately 70%, but by day 12, expression of all three genes rebounded to control levels. These results suggest that expression of *catalase* and *sod-2* are regulated by the transcription factor foxo,

3.4 dsCatalase significantly increases degeneration of follicles in diapausing females

RNAi efficiency was first assessed by qRT-PCR. In contrast to the relatively high induction of *catalase* in ds β -gal injected mosquitoes, only 36 % of *catalase* mRNA was detected in dsCatalase injected, diapausing (D) mosquitoes one week after injection, thus indicating that injection of dsCatalase successfully reduced induction of the *catalase* gene (Fig. 3). The *rpl19* gene was used as an internal control. qRT-PCR analysis of *rpl19* transcript levels one week after injection revealed no significant differences among the treatments (Fig 3).

One week after adult eclosion the proportion of females with ovarian follicle cells in the process of degeneration was 15.6–17.2% in D untreated females, ds β -gal injected females, and in dsSOD-2 injected females (Table 1). By contrast, when dsCatalase was injected into D females the proportion of females with degenerating follicles greatly increased (63.3%) and was significantly different from that observed in untreated controls (Table 1). Neutral Red staining was detected in only a few regions of follicles from D untreated females, ds β -gal injected controls and dsSOD-2 injected mosquitoes. When follicles were examined under ultraviolet light, after staining with Acridine Orange, they autofluoresced (Fig. 4B). Degenerating follicles, detected by intense staining with Neutral Red, were observed in dsCatalase injected mosquitoes (Fig. 4C), and Acridine Orange staining of the same ovaries revealed condensed nuclear chromatin (Fig. 4D).

3.5 Reduced survival of diapausing *C. pipiens* in response to dsCatalase and dsSOD-2

While > 90% of ds β -gal injected, diapausing mosquitoes survived 10 days after injection, only 62.5% of the dsCatalase injected females survived 5 days after injection, and 55 % survived for 10 day (Fig. 5). This result suggests that, at least in early diapause, reduced expression levels of *catalase* significantly increased mortality of *C. pipiens*, possibly a consequence of reduced antioxidant activity. Interestingly, although we did not detect a significant difference in survival for dsSOD-2 injected females at 5 days, only 55 % of the mosquitoes were alive 10 days after injection (Fig. 5). These different patterns of mortality may indicate different functional role for these two antioxidant enzymes in this system.

4. Discussion

Among the five antioxidant-related genes that we investigated, three *superoxide dismutases* (SODs), a *catalase* and a *glutathione peroxidase* (Gpx), two (*catalase* and *sod-2*) were highly up-regulated in diapausing females one week after adult eclosion in comparison to nondiapausing females of the same age. When we knocked down *sod-2* or *catalase* in diapausing females, mortality increased dramatically, an effect similar to the increase we observed when we knocked down *foxo* (Sim and Denlinger, 2008). In this experiment, we evaluated the impact of suppression of *foxo* by RNAi on the expression of *catalase* and *sod-2* and report that suppression of *foxo* suppressed transcript levels of *sod-2* and *catalase* as well. Although promoter analysis of the candidate downstream genes of *foxo* is needed, this result suggests that transcriptional regulation of *sod-2* and *catalase* may be under control of this key transcriptional factor, FOXO. Our previous work suggested a critical role for FOXO in initiating the diapause syndrome in this mosquito (Sim and Denlinger, 2008), and this study now suggests that *catalase* and *sod-2* are members of the downstream gene network presiding over the diapause program (Fig. 6).

When *catalase* and *sod-2* were suppressed by RNAi, the proportion of degenerating follicles increased significantly in dsCatalase treated diapausing mosquitoes, but there was not a significant change in dsSOD-2 treated females. Interestingly, survival of dsSOD-2 treated mosquitoes dropped rapidly 10 days after injection, similar to that observed in dsCatalase treated diapausing mosquitoes. These distinctions suggest that *catalase* and *sod-2* may be regulated in tissue-specific or temporally-specific modes, and functions of these two genes may be linked to different challenges experienced during the overwintering diapause. In another mosquito, *An. gambiae*, a blood meal elicits different expression patterns for genes encoding various antioxidant enzymes, including *catalase*, *superoxide dismutases* and *peroxidase*, and distinct expression patterns are also noted in different tissues such as the fat body, midgut and ovary (DeJong et al., 2007). Both *catalase* mRNA and enzymatic activity increase significantly as the ovaries of *An. gambiae* develop, and suppression of *catalase* significantly reduces fecundity (DeJong et al., 2007), a result that is congruent with our observations that *catalase* affects the status of the ovarian follicles in *C. pipiens*.

Ovaries from diapausing females with RNAi-suppressed *catalase* expression displayed condensed nuclear chromatin that was conspicuous in follicle cells with Acridine Orange staining, a feature characteristic of apoptosis. This pattern was not observed in diapausing mosquitoes treated with either dsRNA of *sod-2* or *β -gal*. The hormonal balance between JH and ecdysone is suggested to influence insect follicle degeneration involving apoptosis (Capella and Hartfelder, 1998; Soller et al., 1999). In the honey bee, JH inhibits induction of apoptosis in female gonads during larval development, but the opposite is observed in aphids, where JH induces ubiquitin-dependent programmed cell death (Kobayashi and Ishikawa, 1994). In the mosquito *C. pipiens*, suppression of JH synthesis is a key factor initiating diapause (Meola and Petralia, 1980). The shut-down in JH synthesis is thought to evoke many features of the diapause syndrome including ovarian arrest and enhanced stress tolerance. An application of JH or a JH analog to the diapausing mosquito breaks the diapause, promoting ovarian development and concurrently reducing stress tolerance (Sim and Denlinger, 2008; Spielman, 1974). Breaking diapause thus makes the mosquito more vulnerable to oxidative stress, perhaps by increasing the metabolic rate and by inactivating the antioxidant enzymes. Which of these responses are directly regulated by JH and which may be a consequence of diapause termination remains to be resolved.

Why *sod-2* is the only SOD gene involved in the diapause response is not immediately clear, but different functions are linked to different SODs as well as different cellular localizations (Crapo et al., 1992; Sturtz et al., 2001). Protein domain searches using pfam indicate that SOD-1 in *C. pipiens* is highly similar to manganese-iron (Mn-Fe) superoxide dismutase, which is localized in mitochondria and peroxisomes. SOD-2 and -3 both have high similarities to copper-zinc (Cu-Zn) superoxide dismutases, which are mainly localized in the cytoplasm or are extracellular. Interestingly, in the nematode *Caenorhabditis elegans* orthologs of SOD-2 and -3 transcripts are significantly up-regulated in the dauer state, a stage equivalent to diapause in insects (Honda et al., 2008). Further examination of developmental and tissue-specific expression patterns of the genes encoding each of these enzymes may help explain why SOD-2 is specifically activated in early diapause.

We've recently shown that the insulin/FOXO signaling pathway has an important role in initiating and maintaining diapause in *C. pipiens* (Sim and Denlinger, 2008). This signaling pathway is well conserved in dormancy regulation in model organisms including the nematode *C. elegans* and the fly *D. melanogaster*. In the fly, dFOXO has a critical role in systemic regulation of antioxidant enzymes. This regulation occurs through the insulin/FOXO signaling pathway in the insulin producing cells (IPCs) (Hwangbo et al., 2004; Kops et al., 2002). dFOXO activation subsequently increases stress tolerance through up-regulation of SODs (Mattila et al., 2009). This genetic regulation of antioxidant enzymes by

FOXO is also noted in *C. elegans*. Dauer larvae, the diapause-like stage in nematodes, increase expression of protective enzymes, SODs and catalase, as well as heat shock proteins (Dalley and Golomb, 1992). In addition, several genome-wide studies indicate that detoxification/stress response genes are among the most common group of genes regulated by FOXO (Gershman et al., 2007; Murphy et al., 2003; Oh et al., 2006). In both *C. elegans* and *D. melanogaster* additional work is needed to verify the function of these candidate genes through explicit genetic dissection. We anticipate that these genome-wide approaches will help clarify the detailed mechanisms involved in regulating many facets of the diapause syndrome in mosquitoes as well.

In conclusion, we have demonstrated that genes encoding two antioxidant enzymes, *catalase* and *sod-2*, offer a protective role in diapausing mosquitoes. Catalase, in particular, plays a key role in protecting arrested ovaries from potential oxidative damage. In addition, we propose that the genes encoding these two antioxidant enzymes are under the control of FOXO, a key transcription factor involved in regulating the diapause program of *C. pipiens*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

SOD	superoxide dismutase
Gpx	glutathione peroxidase
dsSOD	double strand RNA of <i>C. pipiens</i> superoxide dismutase
dsCatalase	double strand RNA of <i>C. pipiens</i> catalase
dsβ-gal	double strand RNA of β-galactosidase
qRT-PCR	quantitative real-time PCR
RNAi	RNA interference
ND	nondiapause
D	diapause
JH	juvenile hormone

References

- Arensburger P, Megy K, Waterhouse RM, Abrudan J, Amedeo P, Antelo B, Bartholomay L, Bidwell S, Caler E, Camara F, Campbell CL, Campbell KS, Casola C, Castro MT, Chandramouliswaran I, Chapman SB, Christley S, Costas J, Eisenstadt E, Feschotte C, Fraser-Liggett C, Guigo R, Haas B, Hammond M, Hansson BS, Hemingway J, Hill SR, Howarth C, Ignell R, Kennedy RC, Kodira CD, Lobo NF, Mao CH, Mayhew G, Michel K, Mori A, Liu NN, Naveira H, Nene V, Nguyen N, Pearson MD, Pritham EJ, Puiu D, Qi YM, Ranson H, Ribeiro JMC, Roberston HM, Severson DW, Shumway M, Stanke M, Strausberg RL, Sun C, Sutton G, Tu ZJ, Tubio JMC, Unger MF, Vanlandingham DL, Vilella AJ, White O, White JR, Wondji CS, Wortman J, Zdobnov EM, Birren B, Christensen BM, Collins FH, Cornel A, Dimopoulos G, Hannick LI, Higgs S, Lanzaro GC, Lawson D, Lee NH, Muskavitch MAT, Raikhel AS, Atkinson PW. Sequencing of *Culex*

- quinquefasciatus* establishes a platform for mosquito comparative genomics. *Science*. 2010; 330:86–88. [PubMed: 20929810]
- Barr AR. The distribution of *Culex p. pipiens* and *Culex p. quinquefasciatus* in North America. *American Journal of Tropical Medicine and Hygiene*. 1957; 6:153–165. [PubMed: 13403135]
- Bashan N, Kovsan J, Kachko I, Ovadia H, Rudich A. Positive and negative regulation of insulin signaling by reactive oxygen and nitrogen species. *Physiological Reviews*. 2009; 89:27–71. [PubMed: 19126754]
- Bell WJ, Bohm MK. Oosorption in insects. *Biological Reviews of the Cambridge Philosophical Society*. 1975; 50:373–396. [PubMed: 1106789]
- Capella IC, Hartfelder K. Juvenile hormone effect on DNA synthesis and apoptosis in caste-specific differentiation of the larval honey bee (*Apis mellifera* L.) ovary. *Journal of Insect Physiology*. 1998; 44:385–391. [PubMed: 12770156]
- Christophers S. The development of the egg follicle in Anophelines. *Paludism*. 1911; 1:73–88.
- Clements AN, Boocock MR. Ovarian development in mosquitos - stages of growth and arrest, and follicular resorption. *Physiological Entomology*. 1984; 9:1–8.
- Crapo JD, Oury T, Rabouille C, Slot JW, Chang LY. Copper, zinc superoxide dismutase is primarily a cytosolic protein in human cells. *Proceedings of the National Academy of Sciences*. 1992; 89:10405–10409.
- Dalley BK, Golomb M. Gene expression in the *Caenorhabditis elegans* dauer larva: developmental regulation of Hsp90 and other genes. *Developmental Biology*. 1992; 151:80–90. [PubMed: 1577199]
- DeJong RJ, Miller LM, Molina-Cruz A, Gupta L, Kumar S, Barillas-Mury C. Reactive oxygen species detoxification by catalase is a major determinant of fecundity in the mosquito *Anopheles gambiae*. *Proceedings of the National Academy of Sciences*. 2007; 104:2121–2126.
- Gershman B, Puig O, Hang L, Peitzsch RM, Tatar M, Garofalo RS. High-resolution dynamics of the transcriptional response to nutrition in *Drosophila*: a key role for dFOXO. *Physiological Genomics*. 2007; 29:24–34. [PubMed: 17090700]
- Giannakou ME, Partridge L. Role of insulin-like signaling in *Drosophila* lifespan. *Trends in Biochemical Sciences*. 2007; 32:180–188. [PubMed: 17412594]
- Honda Y, Tanaka M, Honda S. Modulation of longevity and diapause by redox regulation mechanisms under the insulin-like signaling control in *Caenorhabditis elegans*. *Experimental Gerontology*. 2008; 43:520–529. [PubMed: 18406553]
- Hopwood JA, Ahmed AM, Polwart A, Williams GT, Hurd H. Malaria-induced apoptosis in mosquito ovaries: A mechanism to control vector egg production. *Journal of Experimental Biology*. 2001; 204:2773–2780. [PubMed: 11683433]
- Hwangbo DS, Gershman B, Tu MP, Palmer M, Tatar M. *Drosophila* dFOXO controls lifespan and regulates insulin signaling in brain and fat body. *Nature*. 2004; 429:562–566. [PubMed: 15175753]
- Kim M, Robich RM, Rinehart JP, Denlinger DL. Upregulation of two actin genes and redistribution of actin during diapause and cold stress in the northern house mosquito, *Culex pipiens*. *Journal of Insect Physiology*. 2006; 52:1226–1233. [PubMed: 17078965]
- Kobayashi M, Ishikawa H. Involvement of juvenile-hormone and ubiquitin-dependent proteolysis in-flight muscle breakdown of alate aphid (*Acyrtosiphon pisum*). *Journal of Insect Physiology*. 1994; 40:107–111.
- Kops GJ, Dansen TB, Polderman PE, Saarloos I, Wirtz KW, Coffey PJ, Huang TT, Bos JL, Medema RH, Burgering BM. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. *Nature*. 2002; 419:316–321. [PubMed: 12239572]
- Lee R, Hench J, Ruvkun G. Regulation of *C. elegans* DAF-16 and its human ortholog FKHL1 by the daf-2 insulin-like signaling pathway. *Current Biology*. 2001; 11:1950–1957. [PubMed: 11747821]
- Mattila J, Bremer A, Ahonen L, Kostiaainen R, Puig O. *Drosophila* Foxo regulates organism size and stress resistance through an adenylate cyclase. *Molecular and Cellular Biology*. 2009; 29:5357–5365. [PubMed: 19651894]
- McElwee J, Bubb K, Thomas JH. Transcriptional outputs of the *Caenorhabditis elegans* forkhead protein DAF-16. *Aging Cell*. 2003; 2:111–121. [PubMed: 12882324]

- Meola R, Petralia R. Juvenile hormone induction of biting behavior in *Culex* mosquitoes. *Science*. 1980; 209:1548–1550. [PubMed: 17745968]
- Mitchell CJ. Differentiation of host-seeking behavior from blood-feeding behavior in overwintering *Culex pipiens* (Diptera: Culicidae) and observations on gonotrophic dissociation. *Journal of Medical Entomology*. 1983; 20:157–163. [PubMed: 6842524]
- Mitchell CJ, Briegel H. Fate of the blood meal in force-fed, diapausing *Culex pipiens* (Diptera: Culicidae). *Journal of Medical Entomology*. 1989a; 26:332–341. [PubMed: 2769714]
- Mitchell CJ, Briegel H. Inability of diapausing *Culex pipiens* (Diptera: Culicidae) to use blood for producing lipid reserves for overwinter survival. *Journal of Medical Entomology*. 1989b; 26:318–326. [PubMed: 2769712]
- Murphy C, McCarroll S, Bargmann C, Fraser A, Kamath R, Ahringer J, Li H, Kenyon C. Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*. *Nature*. 2003; 424:277–283. [PubMed: 12845331]
- Oh S, Mukhopadhyay A, Dixit B, Raha T, Green M, Tissenbaum H. Identification of direct DAF-16 targets controlling longevity, metabolism and diapause by chromatin immunoprecipitation. *Nature Genetics*. 2006; 38:251–257. [PubMed: 16380712]
- Rinehart JP, Robich RM, Denlinger DL. Enhanced cold and desiccation tolerance in diapausing adults of *Culex pipiens*, and a role for Hsp70 in response to cold shock but not as a component of the diapause program. *Journal of Medical Entomology*. 2006; 43:713–722. [PubMed: 16892629]
- Robich RM, Denlinger DL. Diapause in the mosquito *Culex pipiens* evokes a metabolic switch from blood feeding to sugar gluttony. *Proceedings of the National Academy of Sciences*. 2005; 102:15912–15917.
- Robich RM, Rinehart JP, Kitchen LJ, Denlinger DL. Diapause-specific gene expression in the northern house mosquito, *Culex pipiens* L., identified by suppressive subtractive hybridization. *Journal of Insect Physiology*. 2007; 53:235–245. [PubMed: 17098250]
- Sanburg LL, Larsen JR. Effect of photoperiod and temperature on ovarian development in *Culex pipiens pipiens*. *Journal of Insect Physiology*. 1973; 19:1173–1190. [PubMed: 4708144]
- Sim C, Denlinger DL. Insulin signaling and FOXO regulate the overwintering diapause of the mosquito *Culex pipiens*. *Proceedings of the National Academy of Sciences*. 2008; 105:6777–6781.
- Sim C, Denlinger DL. A shut-down in expression of an insulin-like peptide, ILP-1, halts ovarian maturation during the overwintering diapause of the mosquito *Culex pipiens*. *Insect Molecular Biology*. 2009a; 18:325–332. [PubMed: 19523064]
- Sim C, Denlinger DL. Transcription profiling and regulation of fat metabolism genes in diapausing adults of the mosquito *Culex pipiens*. *Physiological Genomics*. 2009b; 39:202–209. [PubMed: 19706691]
- Soller M, Bownes M, Kubli E. Control of oocyte maturation in sexually mature *Drosophila* females. *Developmental Biology*. 1999; 208:337–351. [PubMed: 10191049]
- Spielman A. Studies on autogeny in *Culex pipiens* populations in nature. I. reproductive isolation between autogenous and anautogenous populations. *American Journal of Hygiene*. 1964; 80:175–183. [PubMed: 14215828]
- Spielman A. Studies on autogeny in natural populations of *Culex pipiens*. II. seasonal abundance of autogenous and anautogenous populations. *Journal of Medical Entomology*. 1971; 8:555–561. [PubMed: 5160262]
- Spielman A. Effect of synthetic juvenile hormone on ovarian diapause of *Culex pipiens* mosquitoes. *Journal of Medical Entomology*. 1974; 11:223–225. [PubMed: 4851698]
- Sturtz LA, Diekert K, Jensen LT, Lill R, Culotta VC. A fraction of yeast Cu, Zn-superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria. A physiological role for SOD1 in guarding against mitochondrial oxidative damage. *Journal of Biological Chemistry*. 2001; 276:38084–38089. [PubMed: 11500508]
- Tatar M, Kopelman A, Epstein D, Tu M, Yin C, Garofalo R. A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function. *Science*. 2001; 292:107–110. [PubMed: 11292875]

- Williams K, Busto M, Suster M, So A, Ben-Shahar Y, Leever S, Sokolowski M. Natural variation in *Drosophila melanogaster* diapause due to the insulin-regulated PI3-kinase. *Proceedings of the National Academy of Sciences*. 2006; 103:15911–15915.
- Wilson, AP. Cytotoxicity and viability assays. In: Freeshney, RI., editor. *Animal cell culture*. IRL Press; Oxford: 1990. p. 263-304.

Fig. 1.

Quantitative real-time PCR showing expression of *catalase*, *sod-1*, *-2*, *-3* and *gpx* in *C. pipiens* females, 3 days and 1 week after adult eclosion. D (black) = programmed by short daylength for diapause, ND (white) = programmed by long daylength for nondiapause. Both groups were maintained at 18°C. Bars (mean $\pm$ s.e., n=3) with asterisks (*) indicate significant differences at $P < 0.05$, t-test.

Fig. 2.

RNAi targeted against *foxo* also suppresses expression of *catalase* and *sod-2* genes in *C. pipiens* females, 1 and 3 days after adult eclosion. *Catalase* and *sod-2* transcript levels in females injected with dsFOXO (gray bars) were compared with the double-strand RNA of β -galactosidase (ds β -gal) controls (white bars). Bars (mean $\pm$ s.e., n=3) with asterisks (*) indicate significant differences at $P < 0.05$, t-test

Fig. 3.

RNA interference efficiency in *catalase* and *sod-2* knocked-down mosquitoes that were programmed by short daylength for diapause. *Catalase* and *sod-2* transcript levels in females injected with dsCatalase (gray bars) or dsSOD-2 (black bars) were compared with the ds β -gal controls (white bars). Expression levels were measured by qRT-PCR 1 week after RNAi injection. Ribosomal protein large subunit 19 (*rpl19*) was used to confirm equal loading. Bars (mean $\pm$ s.e., n=3) with asterisks (*) indicate significant differences at $P < 0.05$, t-test.

Fig. 4. Primary follicles from diapausing females, prepared with neutral red and acridine orange dyes 7 days after eclosion under white light (A) and GFP filter (B). Primary follicles from double-strand RNA of catalase (dsCatalase) under white light (C) and GFP filter (D). These follicles were in the process of apoptosis. Scale bar = 50 μ m.

Fig. 5.

The relative percentages (mean $\pm$ s.e., $n=4$) of surviving mosquitoes after injection of dsRNA targeting against β -gal (control, ds- β -gal), *catalase* (dsCatalase) and *sod-2* (dsSOD-2). (*) indicate significant differences at $P = 0.05$, t-test. $N = 4$ groups of 20 individuals for each data point. White boxes = 1 days after adult dsRNA injection (1 d.p.i), Gray boxes = 5 days (5 d.p.i), and Black boxes = 10 days (10 d.p.i.).

Table 1

Proportion of diapausing *C. pipiens* with ovarian follicles in a state of degeneration following an injection of dsCatalase, dsSOD-2 or ds β -gal.

dsRNA injection	N	Females with degenerating follicles (%)
Programmed for diapause		
untreated control	32	15.6
ds β -gal	29	17.2
dsCatalase	30	63.3*
dsSOD-2	31	16.1

* Asterisks indicate significant difference from untreated controls (χ^2 -goodness of fit test at $P < 0.05$ and d.f.=1).