

Ovarian Dual Oxidase (Duox) Activity Is Essential for Insect Eggshell Hardening and Waterproofing*

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Background: Hardening of the insect eggshell is due to peroxidases that promote protein cross-linking via H₂O₂, from an unknown source.

Results: In *Rhodnius prolixus*, this H₂O₂ is produced by Duox, and it also contributes to waterproofing, preventing desiccation.

Conclusion: H₂O₂ from Duox has a double role in protecting the *Rhodnius* eggshell.

Significance: Duox activity is essential to insect reproduction.

In insects, eggshell hardening involves cross-linking of chorion proteins via their tyrosine residues. This process is catalyzed by peroxidases at the expense of H₂O₂ and confers physical and biological protection to the developing embryo. Here, working with *Rhodnius prolixus*, the insect vector of Chagas disease, we show that an ovary dual oxidase (Duox), a NADPH oxidase, is the source of the H₂O₂ that supports dityrosine-mediated protein cross-linking and eggshell hardening. RNAi silencing of Duox activity decreased H₂O₂ generation followed by a failure in embryo development caused by a reduced resistance to water loss, which, in turn, caused embryos to dry out following oviposition. Phenotypes of *Duox*-silenced eggs were reversed by incubation in a water-saturated atmosphere, simultaneous silencing of the *Duox* and catalase genes, or H₂O₂ injection into the female hemocoel. Taken together, our results show that Duox-generated H₂O₂ fuels egg chorion hardening and that this process plays an essential role during eggshell waterproofing.

The insect eggshell, or chorion, was described by Beament (1) as “that part of the egg lying outside the oocyte cell membrane, which is secreted by the follicle.” It is a multilayered structure that confers physical and biological protection to the embryo during development. Although chorion hardening of *Aedes aegypti* eggs takes place only after oviposition (2), in other insects, such as *Drosophila melanogaster* and *Rhodnius prolixus*, the hardening of the eggshell occurs while the oocytes are still in the ovary (3). Similar to what happens with the body wall cuticle of all insects, the hardening of the eggshell in *D. mel-*

nogaster and *A. aegypti* involves cross-linking of the exochorion proteins, which is accomplished by peroxidases through the oxidation of tyrosine residues and the subsequent formation of di- and trityrosine bonds between the chorion proteins, a process that requires H₂O₂ (4, 5). Although the source has not been identified, Margaritis (6) hypothesized that the H₂O₂ that supports chorion hardening in *D. melanogaster* is likely to be produced by an oxidase present at the apical surfaces of the follicle cells.

Dual oxidase (Duox)² enzymes are NADPH oxidases that present an amino-terminal peroxidase-like domain facing the extracellular side of the plasma membrane that is not found in other types of NADPH oxidases (7). Duox enzymes generate hydrogen peroxide (8) either to fuel their own peroxidase activity (9–11) or to support the activity of other peroxidases, including tyrosine bond formation (12, 13) and several other biological processes (14). Resistance to water loss is thought to be an essential feature of insect eggs and probably was a key adaptation that allowed the marine ancestors of insects to invade the terrestrial environment. Immediately after oviposition, the eggs of *A. aegypti* are susceptible to desiccation and become waterproof only after several hours, when the serosal cuticle is formed. This layer, which is secreted below the endochorion by the serosa, surrounds the embryo (15). By contrast, the waterproofing process in *D. melanogaster* and *R. prolixus* takes place in the ovaries, simultaneously with the hardening of the chorion, during the final stages of oogenesis (16, 17). In the present study, we used *R. prolixus*, a hemipteran that is a vector of Chagas disease, to show that a Duox enzyme present in the ovarian follicular epithelium is the source of the H₂O₂ that fuels the hardening of the chorion, a process that is essential for waterproofing the eggshell.

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This paper is dedicated to the memory of Doctor Alexandre A. Peixoto.

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² The abbreviations used are: Duox, dual oxidase; RpDuox, *R. prolixus* Duox; NOX, NADPH oxidase; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-N,N',N',N'-tetraacetic acid tetrakis(acetoxymethyl ester); qPCR, quantitative PCR; ABM, after the blood meal.

EXPERIMENTAL PROCEDURES

Insects—The insects used were adult mated females taken from a *R. prolixus* colony, maintained at 28 °C and 75% relative humidity, and fed on rabbit blood. When mentioned, the insects were kept in a water-saturated atmosphere (relative humidity >96%) at 28 °C. To avoid bacterial and fungal proliferation, 100 units penicillin, 100 µg of streptomycin, and 2.5 µg of fungizone per ml were added to the water that was used to fill the humid chamber.

Ethics Statement—All animal care and experimental protocols were conducted in accordance with the guidelines of the Committee for Evaluation of Animal Use for Research (Comissão de Avaliação do Uso de Animais em Pesquisa da Universidade Federal do Rio de Janeiro, CAUAP-UFRJ) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocols were approved by the CAUAP-UFRJ under Registry Number IBQM001. Dedicated technicians at the animal facility at the Institute of Medical Biochemistry (UFRJ) carried out all aspects related to rabbit husbandry under strict guidelines to ensure careful and consistent handling of the animals.

Tissues—The salivary glands, heart, Malpighian tubules, anterior midgut, posterior midgut, hindgut, fat body, ovary, and follicular epithelia were dissected from cold-anesthetized insect females in cold Tyrode's solution (137 mM NaCl, 2.68 mM KCl, 1.8 mM CaCl₂, 5.56 mM glucose, 0.32 mM NaH₂PO₄, 1.16 mM NaHCO₃, pH 7.4).

Identification of the Duox and Catalase Genes—A local BLAST search using the cDNA sequences of *Duox*, *NOX5*, and catalase orthologs as queries was used to identify the *Duox* and catalase sequences in a *R. prolixus* 454 transcriptome database. These partial cDNAs were used to identify the *Duox* (RPTMP03545), *NOX5* (RPTMP07634), and catalase (RPTMP07126) full-length transcripts in the *R. prolixus* genome database available at VectorBase.

RpDuox Structure and Phylogenetic Analysis—Transmembrane α -helices were predicted using the TMHMM server v. 2.0, available from the Center for Biological Sequence Analysis, and the amino acid residue hydrophobicity profile was performed according to Kyte and Doolittle (18) and also by alignment with *Duox* sequences from other insects. The positions of peroxidase-like and NADPH oxidase (NOX) domains as well as EF-hand calcium-binding sites were predicted according to Marchler-Bauer *et al.* (19). The *Duox* sequences of *Acyrtosiphon pisum*, *Anopheles gambiae*, and *D. melanogaster* as well as RpDuox were aligned using the Clustalw2 software, available at the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) website.

The predicted amino acid sequences of these proteins were aligned using the program Muscle (20). Phylogenetic analysis, performed using only the NOX domain, which is found in all members of the NOX family, was based on maximum likelihood analysis, which was carried out with the PhyML program (21) using the JTT model of substitution, with the frequencies estimated from the data and a discrete γ distribution with four categories and 500 bootstrap replicates. The following sequences were used in sequence alignments and phylogenetic

analyses. The *Duox* sequences were: RPR, *R. prolixus* (RPTMP03545); HSAD1, *Homo sapiens* Duox1 (gi 20149640); HSAD2, *H. sapiens* Duox2 (gi 132566532); CEL, *Caenorhabditis elegans* (gi 351063525); DRE, *Danio rerio* (gi 115391856); API, *A. pisum* (gi 193650217); DME, *D. melanogaster* (gi 281364292); AME, *Apis mellifera* (gi 328779750); DPU, *Daphnia pulex* (gi 321466984); ISC, *Ixodes scapularis* (gi 241624918); and AGA, *A. gambiae* (gi 158298988) and PHU, *Pediculus humanus corporis* (gi 242018811). The *NOX5* sequences were: RPR, *R. prolixus* (RPTMP07634); HSA, *H. sapiens* (gi 115527743); DRE, *D. rerio* (gi 189537273); API, *A. pisum* (gi 328705704); TCA, *Tribolium castaneum* (gi 189239162); DME, *D. melanogaster* (gi 161077140); AME, *A. mellifera* (gi 151427582); and PHU, *P. humanus corporis* (gi 242015786) and AGA, *A. gambiae* (gi 151427580). The *NOX1–4* sequences were: HSA1, *H. sapiens* NOX1 (gi 148536873); HSA2, *H. sapiens* NOX2 (gi 6996021); and HSA3, *H. sapiens* NOX3 (gi 11136626) and HSA4, *H. sapiens* NOX4 (gi 8393843).

RNA Extraction, Conventional PCR, and qPCR—Total RNA was extracted from tissues using TRIzol (Invitrogen) according to the manufacturer's protocol. RNA was treated with RNase-free DNase I (Fermentas International Inc., Burlington, Canada), and cDNA was synthesized using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA from salivary glands, heart, Malpighian tubules, anterior midgut, posterior midgut, hindgut, fat body, and ovary were PCR-amplified using the PCR master mix (Fermentas International Inc.), and the same primers were used for qPCR (described below). The fragments were separated by agarose gel electrophoresis (2% w/v), and their sizes were compared with GeneRuler™ 100 bp Plus DNA ladder fragments (Fermentas International Inc.). qPCR was performed on a StepOnePlus real-time PCR system (Applied Biosystems) using the Power SYBR Green PCR master mix (Applied Biosystems). The comparative *C_t* method (22) was used to compare gene expression levels. The *R. prolixus* 18 S rRNA gene was used as an endogenous control (23). The primer pairs used for the amplification of *Duox* and 18 S rRNA cDNA fragments for both conventional and real-time PCR, named DuoxRt and 18sRt, respectively, were *DuoxRt*, forward 5'-TTGTGTTTCGCACAT-CCAAC-3' and reverse 5'-GGTCCAACGAAAATATCCAAA-3'; *18sRt*, forward, 5'-TGTCGGTGTAACTGGCAT-GT-3 and reverse, 5'-TCGGCCAACAAAAGTACACA-3'.

Hydrogen Peroxide Measurement—Measurement of H₂O₂ production by whole ovaries or by dissected follicular epithelia was performed by incubating tissues for 60 min at 25 °C in Tyrode's solution with 40 µM Amplex Red and 0.08 units/µl of horseradish peroxidase (24). After incubation, the ovaries or epithelia were spun, and the supernatant was collected. Fluorescence (excitation, 530 nm; emission, 590 nm) was measured with a microplate reader, the SpectraMax M5 (Molecular Devices). Because *Duox* activity depends on calcium, Ca²⁺-dependent H₂O₂ generation was evaluated by preincubation of the ovaries and follicular epithelia in the presence or absence of 1 µM ionomycin, 10 µM BAPTA/AM, or both for 10 min at 28 °C. Inhibition of *Duox* activity was achieved via preincubation with 1 µM diphenyleiodonium. The fluorescence of non-

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specific (H_2O_2 -independent) oxidation of Amplex Red was evaluated in the absence of HRP and used as a blank.

RNAi Experiments—A 404-bp fragment from the *Duox* gene and a 453-bp fragment from the catalase gene were amplified from reverse-transcribed RNAs extracted from *R. prolixus* ovaries using the primer pairs DuoxDs1 and CatDs1, respectively. The amplification products were subjected to nested PCR with an additional pair of primers (DuoxDs2 and CatDs2) that included the T7 promoter sequence in each fragment. The primers mentioned above were *DuoxDs1*, forward, 5'-ATGG-GTAATCCTGCGTTGAG-3' and reverse, 5'-CCGTCTTTT-GATTCCAGCAT-3'; *CatDs1*, forward, 5'-GGAGCGTTGCG-TTACTTTGA-3' and reverse, 5'-GCAAGTTTCACCTCG-GTCAT-3'; *DuoxDs2*, forward, 5'-TAATACGACTCACTAT-AGGGATGGGTAATCCTGCGTTGAG-3' and reverse, 5'-TAATACGACTCACTATAGGGCCGTCTTTTGATTCCA-GCAT-3'; *CatDs2*, forward, 5'-TAATACGACTCACTATAG-GGGGAGCGTTGCGTTACTTTGA-3' and reverse, 5'-TAA-TACGACTCACTATAGGGGCAAGTTTCACCTCGGTCAT-3'. The nested PCRs generated 444- and 493-bp fragments of *Duox* and catalase, respectively. These fragments were used as a template to synthesize double-stranded RNA (dsRNA) specific for *Duox* (dsDuox) and catalase (dsCat) using the MEGAscript RNAi kit (Ambion, Austin, TX) according to the manufacturer's protocol. An unrelated dsRNA (dsMal) specific for the *Escherichia coli* MalE gene (Gene ID: 948538) was used as a control for the off-target effects of dsRNA. The Mal fragment was amplified from the Litmus 28i-mal plasmid (New England Biolabs) with a single primer (T7, 5'-TAATACGACTCAC-TATAGGG-3') specific for the T7 promoter sequence that is on both sides of the MalE sequence. For gene silencing experiments, cold-anesthetized adult females were injected in the hemocoel with 1 μ l of sterile distilled water containing 1 mg/ml dsRNA using a 5- μ l Hamilton syringe. Six days after dsRNA injection, the insects were fed with rabbit blood.

Oviposition and Eclosion Ratios—After a blood meal, the females were individually separated into vials and kept at 28 °C and 75% relative humidity. After completion of oviposition, the number of eggs laid by each female was counted. The eclosion ratios were calculated by dividing the number of hatched first instar nymphs by the number of eggs laid by each female.

Eggshell Fluorescence—Eggshells were photographed after eclosion with an Olympus MVX10 macroview fluorescence microscope equipped with a Olympus DP-72 color CCD camera without filters, an external LED white light source for bright field imaging, and a filter set for dityrosine fluorescence (4900 ET - DAPI - EX D350/50X; BS 400DCL; EM ET460/50Chroma). Comparison of fluorescence levels between the distinct systems was performed using the same objectives and exposure times. For fluorescence quantification, pictures of three groups of 10 eggs from control and silenced females were analyzed with ImageJ software (24). To calculate the corrected total egg fluorescence (CTEF), we used the following formula: CTEF = integrated density - (area of selected egg \times mean fluorescence of background readings).

Chorion Acid Hydrolysis—Egg chorion obtained from newly laid eggs was subjected to acid hydrolysis to evaluate its dityrosine content using the procedure described by Malencik *et al.*

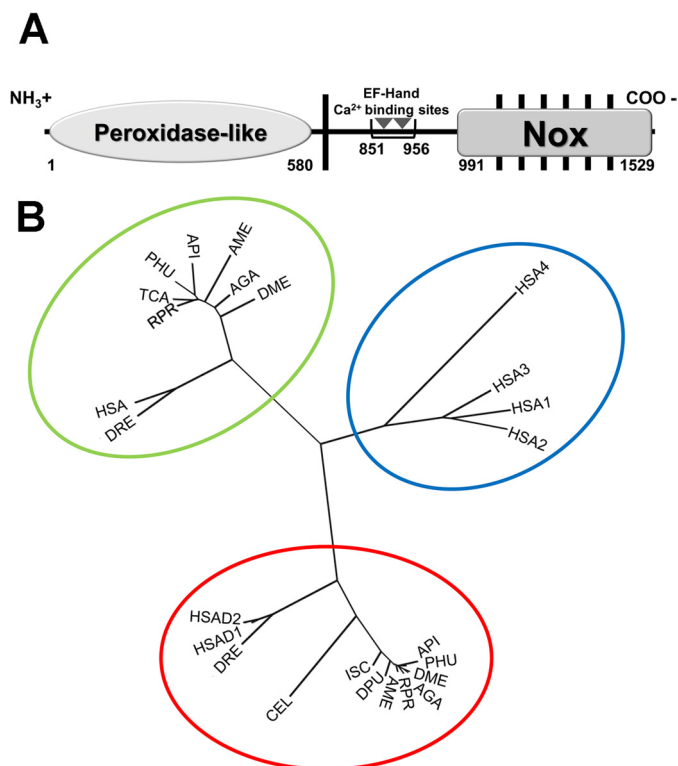


FIGURE 1. Structure and phylogeny relationships of *R. prolixus* dual oxidase. A, the scheme shows the positions of the predicted peroxidase-like and NOX domains, transmembrane α -helices (black bars), and cytosolic EF-hand calcium-binding sites (gray triangles) of *R. prolixus* dual oxidase (RpDuox). B, phylogenetic comparisons between NOX (1–5) sequences and the sequences of Duox NOX domains. The following species were used for tree construction. The Duox sequences were: RPR, *R. prolixus*; HSAD1, *H. sapiens* Duox1; HSAD2, *H. sapiens* Duox2; CEL, *C. elegans*; DRE, *D. rerio*; API, *A. pisum*; DME, *D. melanogaster*; AME, *A. mellifera*; DPU, *D. pulex*; ISC, *I. scapularis*; AGA, *A. gambiae* (gi 158298988) and PHU, *P. humanus corporis*. The NOX5 sequences were: RPR, *R. prolixus*; HSA, *H. sapiens*; DRE, *D. rerio*; API, *A. pisum*; TCA, *T. castaneum*; DME, *D. melanogaster*; AME, *A. mellifera*; PHU, *P. humanus corporis* and AGA, *A. gambiae*. The NOX1–4 sequences were: HSA1, *H. sapiens* NOX1; HSA2, *H. sapiens* NOX2; HSA3, *H. sapiens* NOX3 and HSA4, *H. sapiens* NOX4. Colored ellipses indicate the three NOX clusters, vertebrate specific NOX1–4 (blue), NOX5 (green), and Duox (red).

(25), with some modifications. In three independent experiments, pools of 20 eggs laid by 10 dsMal- or dsDuox-injected females were transferred to 1-ml polypropylene tubes containing 1% Triton X-100 in distilled water, homogenized, sonicated for 10 min, and washed 10 times with distilled water. After sedimentation, chorion fragments were collected, dried under a vacuum, and added to 6 N HCl. The samples were hydrolyzed for 24 h at 110 °C under a vacuum and then dried under a vacuum, resuspended in distilled water, and filtered on Amicon filters (10-kDa cutoff). After being dried under a vacuum, the samples were resuspended in 50 mM sodium phosphate buffer, pH 9.6. For standard dityrosine preparation, 2 mg of tyrosine and 80 μ g of horseradish peroxidase (Sigma) were added to 2 ml of 100 mM Tris-HCl buffer, pH 9.2, containing 0.005% H_2O_2 . After 24 h of incubation at 37 °C, the reaction medium was filtered on Amicon filters (10-kDa cutoff) to remove horseradish peroxidase and dried under a vacuum. The samples were then resuspended in 50 mM sodium phosphate buffer, pH 9.6. Fluorescence excitation spectra (excitation, 280–360 nm; emission, 410 nm) were measured with a Cary Eclipse 100 spec-

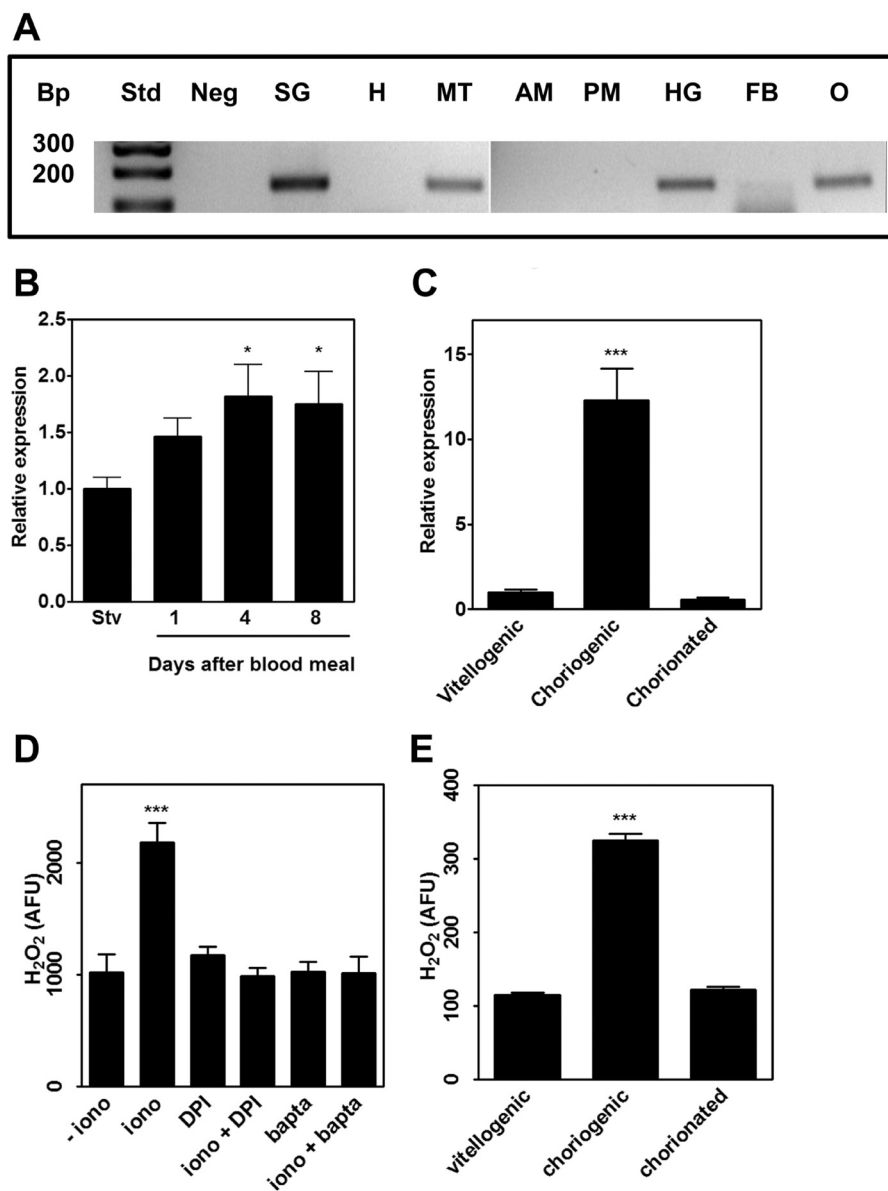


FIGURE 2. **Tissue distribution, expression levels, and activity of RpDuox.** *A*, tissue distribution. *Duox* fragments were PCR-amplified from cDNA samples from salivary glands (SG), heart (H), Malpighian tubules (MT), anterior midgut (AM), posterior midgut (PM), hindgut (HG), fat body (FB), and ovary (O) and separated by 2% agarose gel electrophoresis. (Neg, negative control; Bp, base pair). *B*, *RpDuox* expression levels in ovaries. qPCR assays were performed with ovaries dissected immediately before (starved) or at different days ABM. Eight pairs of ovaries were used for each data set. The levels of *Duox* expression were normalized by the level found in the ovaries of starved (Stv) females. *C*, *RpDuox* expression levels during developmental stages of ovarian follicle epithelia. Epithelia were dissected 7 days ABM from vitellogenic, choriogenic, or chorionated follicles. Nine pools of 10 epithelia were used for each experimental condition. The levels of *Duox* expression were normalized by the level found in vitellogenic follicles. *D*, *Duox* activity in the whole ovary. Generation of H₂O₂ was assayed with HRP/Amplex Red in ovaries from insects dissected 3 days ABM, and fluorescence intensity was plotted as arbitrary fluorescence units (AFU). Five pools of two ovaries were used in each data point. Tissues were preincubated in the presence or absence of ionomycin (iono), diphenyleneiodonium (DPI), or BAPTA/AM. *E*, *Duox* activity in follicular epithelia from vitellogenic, choriogenic, or chorionated follicles. Experimental conditions were the same as used in *D*, except that five pools of 10 follicular epithelia were used for each data point. Data shown are of ionomycin-activated H₂O₂ production. Data shown in all graphics are mean \pm S.E. * $p < 0.05$, *** $p < 0.0001$ (analysis of variance followed by Tukey's multiple comparison test).

trofluorometer (Varian, Palo Alto, CA). Crude eggshell hydrolysate is a complex mixture of substances. To circumvent this problem and gain specificity, the spectra of crude hydrolysate from dsMal samples were subtracted from the spectra of dsDuox samples, a procedure that resulted in a spectral profile that reflected only the differences between the two samples.

Statistical Analysis—All experiments were repeated at least twice. Statistical analysis was performed using Student's *t* test with a 95% confidence interval or a one-way analysis of variance fol-

lowed by Tukey's multiple comparisons post test (GraphPad Prism).

RESULTS

Structural Features, Domains, and Polygenetic Analysis of RpDuox—The presence of a Duox-type NADPH oxidase, initially found in a transcriptome, was confirmed by identification of a complete predicted transcript in the *R. prolixus* genome, hereafter called RpDuox. Fig. 1A shows the characteristic per-

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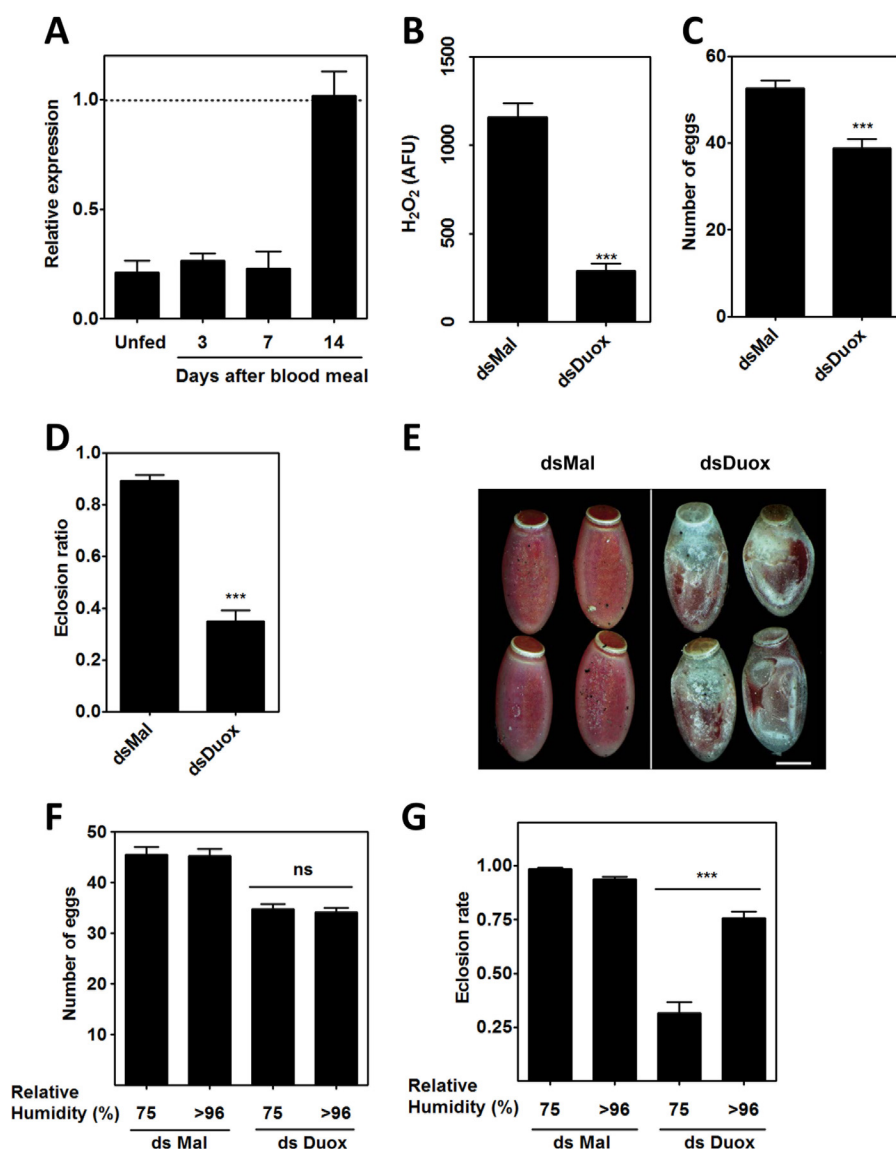


FIGURE 3. *RpDuox* silencing. *R. prolixus* adult females were injected with dsMal or dsDuox 6 days before blood meal. **A**, *RpDuox* expression inhibition in whole ovaries. Ovaries were dissected immediately before (*Unfed*) or at the indicated times after blood meal for qPCR analysis. The *dashed line* represents the expression level shown in the ovaries of control females (dsMal-injected). Five pools of two ovaries were used for each condition. **B**, inhibition of H₂O₂ generation in whole ovaries. Three days after the meal, ovaries were dissected and assayed with HRP/Amplex Red to measure H₂O₂ generation. The data shown represent ionomycin-stimulated H₂O₂ generation plotted as arbitrary fluorescence units (AFU). Five pools of two ovaries were used for each condition. **C** and **D**, effect of *Duox* RNAi on the number of eggs laid (**C**) and eclosion ratios (**D**). Adult females were injected with dsMal (24) and dsDuox (19), respectively, and the number of eggs laid and first instar nymph eclosion ratios were calculated for each female individually. **E**, *RpDuox* silencing impairs embryo development. Bright field representative images of eggs laid by control (DsMal) and *RpDuox*-silenced females at 48 h after oviposition are shown. *Bar scale* = 0.5 mm. **F** and **G**, impairment of embryo development after *RpDuox* silencing is prevented by incubation in an atmosphere of high relative humidity. Eggs were incubated under high humidity (relative humidity >96%). At least nine females were used per condition. In all graphics, the data shown are the mean \pm S.E., and asterisks indicate significantly different values ($p < 0.0001$, Student's *t* test). *ns*, not significant.

oxidase-like, EF-hand, and NOX domains of *RpDuox*, which are conserved among all *Duox* orthologs. A multiple sequence alignment of the *RpDuox* protein sequence against *A. pisum*, *A. gambiae*, and *D. melanogaster* *Duox* sequences showed a remarkable conservation (see Fig. 6). Phylogenetic analysis provided further support for the identification of *RpDuox*, which clustered with other members of the *Duox* group (Fig. 1*B*). Additionally, the *Duox* and *NOX5* branches that were obtained were consistent with known arthropod evolutionary relationships (Fig. 1*B*), including insects arising from Crustacea (26, 27).

Duox Is Expressed in the Ovaries during Choriogenesis—*RpDuox* mRNA was expressed in several of the tissues tested,

including ovaries, salivary glands, Malpighian tubules, hindgut, and fat body (Fig. 2*A*). *RpDuox* expression in the ovary increased after a blood meal (Fig. 2*B*), paralleling the time course of oocyte growth in this insect (28). Evaluation by qPCR of *RpDuox* mRNA in ovarian follicles at distinct developmental stages revealed that the highest expression levels were found in the epithelium of choriogenic follicles and were almost 10 times higher than those in the epithelia from follicles that either were at the vitellogenic growth stage or had already completed choriogenesis (Fig. 2*C*).

Duox enzymes are calcium-dependent NADPH oxidases due to the presence of the EF-hand domain (14). When ovaries dis-

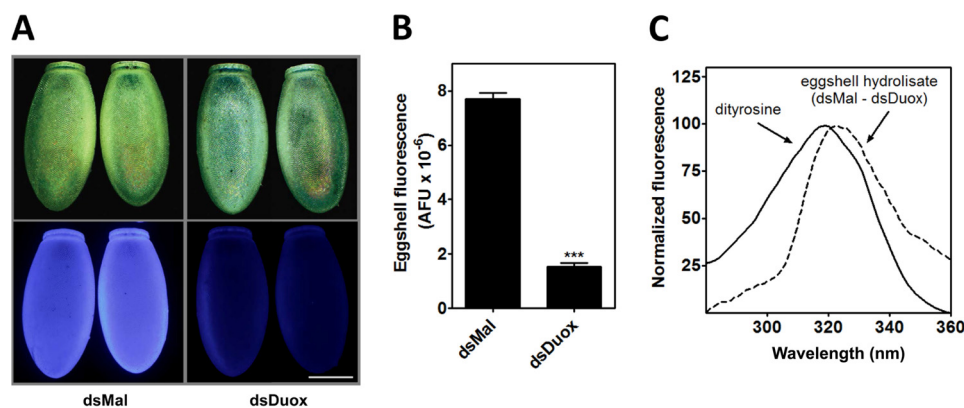


FIGURE 4. The egg chorion of *RpDuoX*-silenced females shows lower levels of dityrosine. *A*, dityrosine fluorescence of egg chorion. Representative images are shown of the eggshells of eggs laid by control (dsMal) and *RpDuoX*-silenced females kept until the hatching at high humidity (described in the previous figure). The bright field images (on the top) were taken with a stereomicroscope using an external white light source, and the fluorescence images were taken with a filter set for intrinsic dityrosine fluorescence (on the bottom). Bar scale = 0.5 mm. *B*, fluorescence emitted by eggshells of dsMal- and dsDuoX-injected females. The total eggshell fluorescence was quantified using ImageJ software. Data shown are the mean \pm S.E. of total eggshell fluorescence calculated for 10 eggshells selected randomly among \sim 400 eggs of each condition. *** $p < 0.0001$, Student's *t* test. AFU, arbitrary fluorescence units. *C*, dityrosine fluorescence excitation spectra of chorion hydrolysates. Eggshells were subjected to acid hydrolysis. After being dried under a vacuum, the samples were resuspended in 50 mM sodium phosphate buffer, pH 9.6. For hydrolysates, the data shown are the normalized spectrum of a dsMal chorion hydrolysate that were subtracted from the spectrum obtained with a dsDuoX chorion hydrolysate sample. The preparation of the dityrosine standard is described under "Experimental Procedures."

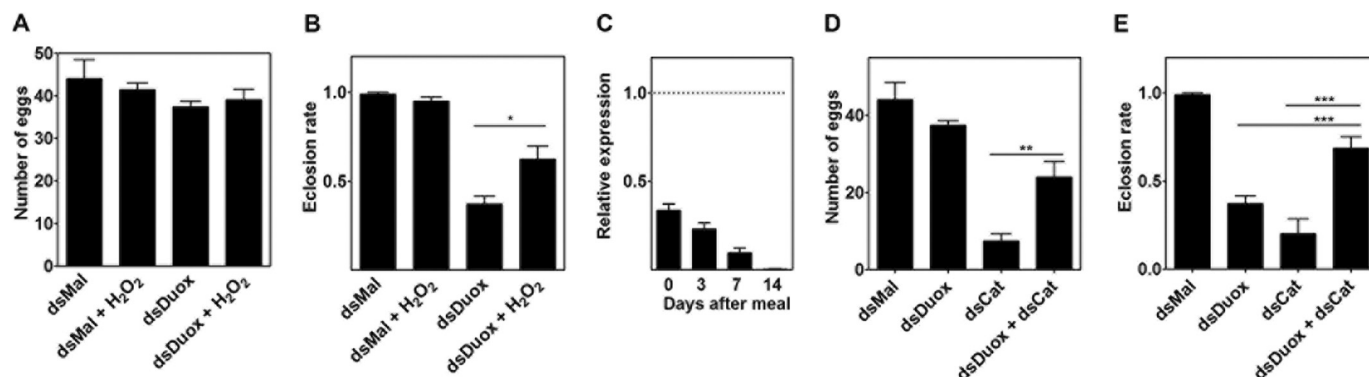


FIGURE 5. H_2O_2 regulates oogenesis and choriogenesis. *A* and *B*, H_2O_2 injection. dsMal- and ds*RpDuoX*-treated females were injected with 5 nmol of H_2O_2 into the hemocoel 24 h ABM, and the number of eggs laid (*A*) and the first instar nymph eclosion rates (*B*) were evaluated. *C* and *D*, catalase knockdown. 6 days before the blood meal, adult females were injected with dsMal, dsDuoX, dsCat, or dsDuoX plus dsCat. *C*, catalase silencing was evaluated by qPCR in whole ovary extracts. *D* and *E*, the number of eggs laid (*D*) and eclosion ratios (*E*) were calculated individually for five females in each condition. In all graphic data shown are the mean \pm S.E., and the asterisks indicate significantly different values between the conditions (*B*, $p = 0.0205$; *D*, $p = 0.0032$; Student's *t* test).

sected from blood-fed females were incubated in the presence of ionomycin, a calcium ionophore that increases intracellular levels of Ca^{2+} , H_2O_2 production by this tissue was markedly stimulated (Fig. 2*D*). Preincubation of ovaries with diphenylethionium (an NADPH oxidase inhibitor) or BAPTA/AM (an intracellular Ca^{2+} chelator) prevented this ionomycin-induced increase in H_2O_2 production, consistent with the presence of a DuoX-type enzyme in this tissue (29). As expected from the increased expression of *RpDuoX* mRNA during chorion formation (Fig. 2*C*), we observed increased ionomycin-stimulated H_2O_2 production in the follicular epithelium during this developmental stage (Fig. 2*E*), suggesting that this enzyme participates in choriogenesis.

***RpDuoX* Silencing Impairs Egg Development**—To investigate the function of *RpDuoX* in the ovaries of *R. prolixus*, we silenced its expression by injecting dsRNA for this gene into the hemocoel of females prior to the blood meal. dsRNA injection reduced *DuoX* mRNA levels in the ovaries by \sim 70% in relation to control females. Efficient silencing was achieved before the blood meal and was maintained until 1 week after the blood

meal (ABM), with *RpDuoX* expression returning to levels similar to the control near the end of oogenesis, 2 weeks ABM (Fig. 3*A*). *RpDuoX* silencing led to a significant reduction of DuoX activity in the ovaries (Fig. 3*B*), modestly reduced the number of eggs laid, and markedly decreased the first instar nymph eclosion ratio (Fig. 3, *C* and *D*). Upon *RpDuoX* silencing, a major fraction of the eggs dried out in the first 48 h after oviposition and did not develop (Fig. 3*E*), suggesting that the permeability barrier of the eggshell was compromised. This conclusion was confirmed by incubating *RpDuoX*-silenced eggs in a high humidity atmosphere, which resulted in eclosion rates similar to those of control eggs (Fig. 3, *F* and *G*).

***RpDuoX* Silencing Inhibits Chorion Protein Cross-linking**—The hardening of the eggshell is known to involve protein cross-linking through peroxidase-mediated oxidation of tyrosine residues (5), which led us to speculate that *RpDuoX* might be the source of the H_2O_2 used in eggshell formation. Consistent with this hypothesis, silencing of *RpDuoX* resulted in the formation of eggshells that showed markedly reduced fluorescence emission under ultraviolet excitation (Fig. 4, *A* and *B*), a

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A

RpDuoX	1	: MVGQRKEQEALFHGLSYKLERQVLELEYIFIYECGYS-----KPYENLKSQEERTHSYVEVQRYDGFNNLNAHPHWGSVESQMKRTRKTPAAYKDGVMMSGQDRSPARRISQL	:	109
ApDuoX	1	: -----FSEKQFSTERQRFEDGFWNNLNAHPQWGSIDSHMTRKTPASYSDGVYMMGQDRSPARRISQL	:	62
LmDuoX	1	: MSVPSAPHQRAESKNRVRPRGQRNKLPLKRLHWPATYGGALLLLISYGLGELGSVHCYKEMYSQTEKRYKQVNNLNAHPDWGSVDSHLVRRKAPPYSDGVYMMGQDRSPARRISQL	:	120
AgDuoX	1	: -----MSHVKEQRYDGFYNNLNAHPDWGAVDNLHTRKAPPASYSDGVYMMGQDRSPARRISQL	:	57
		e s tEKQRYDGFYNNLNAHP WGSvdsH tRK P YsdGVY M G RPS R IS L		
RpDuoX	110	: FMKSGDGLPSIRNRTALLAFGGQVVSSEVVMASSEGCPPIEVHQIPIDKCEMDYDPECKGGKSIPIFHRRARYDPSTGQSPNSPREQVNRVTSWIDGFSVYVSTSEAWTSTMRSYANGSLITTD	:	229
ApDuoX	63	: FMKSGEGLSSLNRTALLAFGGQVVSSEVVMASSEGCPPIKVPHEIEKCEMDYDPECKGGKSIPIFHRRARYDRNTGQSPNTPRQINRVTSWIDGFSVYVSTSEAWNAMRSFNKGTLLSEP	:	182
LmDuoX	121	: FMKSGDGLGSKFNRTALLAFGGQVANEIVMASSEGCPPIEMHRIEIEKCEMDYDRECRGDYIPFHRAAYDRNTGQSPNAPREQINQMTAWIDGFSVYVSTSEAWNAMRSFNKGTLLTEK	:	240
AgDuoX	58	: FMRGTDGLPSMENRTALLAFGGQVTVNEIVMASSEGCPPIEMHRIEIEKCEMDYDRECRGDYIPFHRAAYDRNTGQSPNAPREQINQMTAWIDGFSVYVSTSEAWNAMRSFNKGTLLTEK	:	177
		FM G dGL S NRTALLAFGGQV EivMaSESGCPIe H tEiEKCEMDY Dc K IFFHRA YDr TGQSPN PreQin T WIDGFSVYVSTSEAW namRSf nG LLt		
RpDuoX	230	: TGKLPVKNTRAPLNFQVPHALRTLSPERLFLLDGPRSNQNPALLSFSILWFRWNHKLAEVVKHAPDWPDEEIFHRRARLLIAHQNIIAYEYLPFAFTGIELPEYTYGKLDVHPGVSH	:	349
ApDuoX	183	: TNGFFVRNTRMPLFNNPVPNIRLTLSPERLFLLDGPRSNQNPALLSFGILLFKWNVLADRVQLLEHPDWSDEEVFQARRRVVATLQNIIIAYEYLPFAFTGIELPEYTYGKLDVHPGVSH	:	302
LmDuoX	241	: DGKLPVKNTRMPLFNNPVPVSMKMLSPERLFLLDGPRSNQNPALLSFAILFRWNHTLAQRIRKRVHPDWSDEEIVQARRHTVIAQLNIVVEYLPFAFTGIELPEYTYGKLDVHPGVSH	:	360
AgDuoX	178	: QGTMFVKNTRMPLFNNPVPVSMKMLSPERLFLLDGPRSNQNPALLSFAILFRWNHTLAQRIRKRVHPDWSDEEIVQARRHTVIAQLNIVVEYLPFAFTGIELPEYTYGKLDVHPGVSH	:	297
		g PV NTRMrvPLFNpVp r LSPERLFLLDGPR NQNPALLS F IL rWNN lA Rv HpDwsDEeifQARR via LQNIiAYEYLPFAflg eLP Y GYK D HPG sH		
RpDuoX	350	: VFQSAAFRFGHTLIPPGIYRRDNKCNFRKTPMGNPALRLCSHWDDSDVLESSELEEVLLGMSAQLAEKEDTTLCSVDVRDLFGPNEFSRRDLGALNIMRGRDGLPDYNTVRSFFRLSK	:	469
ApDuoX	303	: VFQSSAFRFGHTMIPPGIYRRDGKCNFRKTPMGNPALRLCSHWDDSDVLESSELEEVLLGMSAQLAEKEDTTLCSVDVRDLFGPNEFSRRDLGALNIMRGRDGLADYNTVRSFFRLSK	:	422
LmDuoX	361	: IFQAAAFRFGHTMIPPGIYRRDGKCNFRKTPMGPVAVRLCSTWDDSGFADTVEEVLMLGASQISEREDPVLCSVDVRDLFGPNEFSRRDLGALNIMRGRDGLPDYNTARESYGLRK	:	480
AgDuoX	298	: MFQAAAFRFGHSLIPPGIYRRDGKCNFRKTPMGPVAVRLCSTWDDSGFADTVEEVLMLGASQISEREDPVLCSVDVRDLFGPNEFSRRDLGALNIMRGRDGLPDYNTARAYRLPK	:	417
		FQ aAFRFGHT IPPGIyRRDG CNfr T Mg PALRLCS WwDsdv svEE GmaSQ aE ED LcSDVRDLFGP EF RRDGLALNIMRGRDGLPDYNT R L		
RpDuoX	470	: HKKWSDINPDLFNSDPTLEKGLSTAYGGSIDNIDLYIGGMLESKDGPGELFAAIIIDQFTRLRSDRFWFENTENGLDKIKEIAEKQVTLWDVIVNATDIEPDSLQKVDVFWSEGDPCCP	:	589
ApDuoX	423	: VEKFDINEELFRFRHDLADKLLKAYG-SVDNVLDYIGGMLESKDGPGELFAAIIIDQFTRLRSDRFWFENTENGLDKIKEIAEKQVTLWDVIVNATDIEPDTIQREVFWSEGDPCCP	:	541
LmDuoX	481	: HKTWTDINPDLFNSDPTLEKGLSTAYGGSIDNIDLYIGGMLESKDGPGELFAAIIIDQFTRLRSDRFWFENTENGLDKIKEIAEKQVTLWDVIVNATDIEPDTIQREVFWSEGDPCCP	:	600
AgDuoX	418	: KKSWRDINPAVFERQPELLLLIKTYDNQLDNVVYVGMLESKDGPGELFAAIIIDQFTRLRSDRFWFENTENGLDKIKEIAEKQVTLWDVIVNATDIEADIEQRDVFHWKGDGDPCCP	:	537
		k w DinP lF P L d L aY Dnvd Y GGMLES PGELF A I dQF R RdaDRWFEN Ng ft eEiae r TLWD IVnStDie d iQ dVf W GDPCP		
		TM1		
RpDuoX	590	: QPAMLNASVLEPCSYLKGDYDFEGSELAYIYACVFLGFVPLVDTAGAGYGVVRLQNRRRRLMRQEEIKSGNKSNSATVDRKMAVREWLHANHRRLLVVKVRFGEAYLHTVDRKGERLRTVN	:	709
ApDuoX	542	: QPGQIEPSTLEPCSYLKGDFYDFEGSELAYIYVCFILGVVPLVDTAGAGYGVVRLQNRRRRLMRQEEIKSGNKSNSATVDRKMAVREWLHANHRRLLVVKVRFGEAYLHTVDRKGERLRTVN	:	661
LmDuoX	601	: QPMQINATELEPCSYLKGDFYDFEGSELAYIYVCFILGVVPLVDTAGAGYGVVRLQNRRRRLMRQEEIKSGNKSNSATVDRKMAVREWLHANHRRLLVVKVRFGEAYLHTVDRKGERLRTVN	:	718
AgDuoX	538	: QPEQLNATELEPCSYLKGDFYDFEGSELAYIYVCFILGVVPLVDTAGAGYGVVRLQNRRRRLMRQEEIKSGNKSNSATVDRKMAVREWLHANHRRLLVVKVRFGEAYLHTVDRKGERLRTVN	:	656
		QP qlna LEPC YL GyDYF GELayIy CvFLGFVp cAGAGY VvRLQN rRr L QEAM N VEM RM REWLHAN RLV V FGPeA TVDRKGERLRT n		
RpDuoX	710	: FKNTDTVTVEESAPERGGKRRMPLVIRVPRDHDVLEFDLTSARRKFKINKLDAFLTSQKHLVILQSGREAMLSKAETKERRQRRLHFFREAYALTFLGKPGERRRRGSAAE-ADGDVV	:	828
ApDuoX	662	: FARCDTITVEESQVDSKRRRPLVIRVPRDHDVLEFDLTSARRKFKINKLDAFLTSQKHLVILQSGREAMLSKAETKERRQRRLHFFREAYALTFLGKPGERRRRGSAAE-ADGDVV	:	776
LmDuoX	719	: LKHVDVVSVEESQVDSKRRRPLVIRVPRDHDVLEFDLTSARRKFKINKLDAFLTSQKHLVILQSGREAMLSKAETKERRQRRLHFFREAYALTFLGKPGERRRRGSAAE-ADGDVV	:	837
AgDuoX	657	: LKHVDVVSVEESQVDSKRRRPLVIRVPRDHDVLEFDLTSARRKFKINKLDAFLTSQKHLVILQSGREAMLSKAETKERRQRRLHFFREAYALTFLGKPGERRRRGSAAE-ADGDVV	:	775
		k D vtVes KKK P L RVP DHDVLE s aRRKf Kle FL hKK Rd mLa AETRRRQRRLHFFREAYALTFLGpGERRR a V		
RpDuoX	829	: MRTSLSRGEFASALGMKDDVFRMFMNIVDKDGRISFQEFLLTVLFSRGTEDDKIRIIFDMCDNDRNGVIDKGELSEMRLSVEIARTTSLSDQHVTTELIDGMFQVAGLEKDFLL	:	948
ApDuoX	777	: MRTSLSKAEFASALGMKADAVFRMFMNIVDKDGRISFQEFLLTVLFSRGTEDDKIRIIFDMCDNDRNGVIDKGELSEMRLSVEIARTTSLSDQHVTTELIDGMFQVAGLEKDFLL	:	896
LmDuoX	838	: MRTSLSKAEFAALGMKPNDFVRMFMNIVDKDGRISFQEFLLTVLFSRGTEDDKIRIIFDMCDNDRNGVIDKGELSEMRLSVEIARTTSLSDQHVTTELIDGMFQVAGLEKDFLL	:	957
AgDuoX	776	: MRTSLSKSEFAALGMKQDDVFRMFMNIVDKDGRISFQEFLETVLFSRGTEDDKIRIIFDMCDNDRNGVIDKGELSEMRLSVEIARTTSLSDQHVTTELIDGMFQVAGLEKDFLL	:	895
		MRTSLSk EFA ALGMK dd FVR MFNIVDKD DGRISFQEF LL TVVLFsRGt DKIRIIFDMCDNDRNGVIDKGELSEM RSLVEIARTTSL D VtelIDGMFQ vGLE K IT		
		TM2		
RpDuoX	949	: YADFKLMMKEYKGFDAVIGLDCRGAQNFLDTSTNVARMTSHIEPIIDDDKHWTETYNVCTVFLLENRQNIYFLFLFYVITIALFVFRFIIHYSFLAEHTDLRHIMGVGIAITRGSAA	:	1068
ApDuoX	897	: YADFKLMMKEYKGFDAVIGLDCRGAQNFLDTSTNVARMTSHVDPDLHDDKRIKSDICVLYLEENRQNIYFLFLFYVITIALFVHQFIYYSFLAEHTDLRHIMGVGIAITRGSAA	:	1016
LmDuoX	958	: YQDFKLMMKEYKGFDAVIGLDCRGAQNFLDTSTNVARMTSHIEPIIDDDKHWTETYNVCTVFLLENRQNIYFLFLFYVITIALFVFRFIIHYSFLAEHTDLRHIMGVGIAITRGSAA	:	1077
AgDuoX	896	: YEDFKLMMKEYKGFDAVIGLDCRGAQNFLDTSTNVARMTSHIEPIIDDDKHWTETYNVCTVFLLENRQNIYFLFLFYVITIALFVFRFIIHYSFLAEHTDLRHIMGVGIAITRGSAA	:	1015
		YDFKLMMKEYKGFDAVIGLDCRGAQNFLDTSTNVARMTSHieP d hw dc TfLEENRQNIYFLFLFYVITi LFVerFIhYSF AEHTDLRHIMGVGIAITRGSAA		
		TM4	TM5	
RpDuoX	1069	: LSFYCSVLLTMSRNLTKLKEFSIQYIPLDISHIYFHKIACTALFFSLHTVGHVNFYHVSTQPLEHVHCLTSEVHFPSDYKPGITFWLQFTITGIGVLLIIVMTIIFVFAHPTIR	:	1188
ApDuoX	1017	: LSFYGLLLTMSRNLTKLKEFSIQYIPLDISHIYFHKIACTALFFSIIHTVGHVNFYHVSTQPLEHVHCLTSEIHFPSDYKPGISFVLFKTIITGIGVLLIIVMTIIFVFAHPTIR	:	1136
LmDuoX	1078	: LSFYCSVLLTMSRNLTKLKEFSIQYIPLDISHIYFHKIACTALFFSLHTVGHVNFYHVSTQSHENLCLTREVHFASDYKPDITFWLQFTITGIGVLLIIVMTIIFVFAHPTIR	:	1197
AgDuoX	1016	: LSFYCSVLLTMSRNLTKLKEFSIQYIPLDISHIYFHKIACTALFFSLHTVGHVNFYHVSTQSHENLCLTREVHFASDYKPDITFWLQFTITGIGVLLIIVMTIIFVFAHPTIR	:	1135
		LSFyCSvLLLTMSRNLTKLKeF IQYIPLDShI FHKIaCTALFFS lHTVGHVnFYHVSTQ Enl CLT EvHf SDYkP ItfWlQftITG TGV LF M iIF FAHPTIR		
		TM6	TM7	FAD binding site
RpDuoX	1189	: KKAYKFFWSAHSYVVLVCLVHGLAKLGPFRFVIFVGGPIIYTLDKVVSCTRYMALDVLTELLPSDVIKIKFYRPPNFYLSGQVRLSCTSFQEEFHSFTLTSAPHENFLSC	:	1308
ApDuoX	1137	: KKAYTFWSTHCLYVAMYLMLLHGLARITGPPFRFVIFVGGPIIIFLTKVVSCTRYMALDVLTELLPSDVIKIKFYRPPNFYLSGQVRLSCTSFQEEFHSFTLTSAPHENFLSC	:	1256
LmDuoX	1198	: KKAYNFWNMHTLYGLVLSLHGLARLTPGFRFVIFVGGPIIYTLDKVVSCTRYMALDVLTELLPSDVIKIKFYRPPNFYLSGQVRLSCTSFQEEFHSFTLTSAPHENFLSC	:	1317
AgDuoX	1136	: KKAYKFFWNAHSYVVLVCLVHGLARLTPGFRFVIFVGGPIIYTLDKVVSCTRYMALDVLTELLPSDVIKIKFYRPPNFYLSGQVRLSCTSFQEEFHSFTLTSAPHENFLSC	:	1255
		KKAY fFw H Lyv lY L L HGLArLTPGPrFw ff GPGI yTLdK VSLRvRYMALDv eT LLPSDVIKIKFYRPPN KYLGSQVrLsCt E HSFTLTSAPHENFLSC		
RpDuoX	1309	: HIRKAGPWTWKLKRNYPDPCNFNAELDQPKIRLEGPFGGNQDQWYFEVAVMVGGGIGVTPYASILNDLVFGTSTNRYSGVCKKVYFLWICPSHSHKFEWFIDVLRDVEKDKVTNVLEIHI	:	1428
ApDuoX	1257	: HIRKAGHWTWKLKRNYPDPSNINPEFHQPKIRLEGPFGGNQDQWYFEVAVMVGGGIGVTPYASILNDLVFGTSTNRYSGVCKKVYFLWICPSHSHKFEWFIDVLRDVEKDKVTNVLEIHI	:	1376
LmDuoX	1318	: HIRKAGPWTWKLKRNYPDPCNYPNPE-DQPKIRIEGPFGGNQDQWYFEVAVMVGGGIGVTPYASILNDLVFGTSTNRYSGVCKKVYFLWICPSHSHKFEWFIDVLRDVEKDKVTNVLEIHI	:	1436
AgDuoX	1256	: HIRKAGPWTWKLKRNYPDPCNYPNPE-DQPKIRIEGPFGGNQDQWYFEVAVMVGGGIGVTPYASILNDLVFGTSTNRYSGVCKKVYFLWICPSHSHKFEWFIDVLRDVEKDKVTNVLEIHI	:	1374
		HIRKAGpWTWKLKRNyDPcN Npe dQPKIR EGPFGGNQDQWYFEVAVMVGGGIGvTPYASILNDLVFGTSTNRYSGV CKKVYFLWICPSHSHKFEWFIDVLRDVEKDKVTNVLEIHI		
		NADPH binding site		
RpDuoX	1429	: FITQFFHFDLRTMLTYICENHFQRLSKTSMFTGLKAVNHFRGPRDMSFLFKVQKHSYVSKIGVFCSCGPRPLTKSVMSACDEVNKRKRLPYFIHFFHFNFG	:	1529
ApDuoX	1377	: FITQFFHFDLRTMLTYICENHFQRLSKTSMFTGLKAVNHFRGPRDMSFLFKVQKHSYVSKIGVFCSCGPRPLTKSVMSACDEVNKRKRLPYFIHFFHFNFG	:	1477
LmDuoX	1437	: FITQFFHFDLRTMLTYICENHFQRLSKTSMFTGLKAVNHFRGPRDMSFLFKVQKHSYVSKIGVFCSCGPRPLTKSVMSACDEVNKRKRLPYFIHFFHFNFG	:	1537
AgDuoX	1375	: FITQFFHFDLRTMLTYICENHFQRLSKTSMFTGLKAVNHFRGPRDMSFLFKVQKHSYVSKIGVFCSCGPRPLTKSVMSACDEVNKRKRLPYFIHFFHFNFG	:	1475
		FITQFFHFDLRTMLTYICENHFQRLSKTSMFTGLKAVNHFRGPRDMSFLFKVQKHSYVSKIGVFCSCGPRPLTKSVMSAC EVNK RKLPYFIHFFHFNFG		

B

HsMPO	256	QQLLDHDLDF	//	400	LAGDTRSSSEMPELT	//	497	AFRYGHTLIQP
HsDuoX1	103	GYHVLSDLVF	//	233	AFYAERGNREPEFLQ	//	326	SEQVCLVTMVP
CeDuoX1	100	QVVAYEIMQS	//	230	MLGDSTRVNEPGLL	//	325	AFRVPHSIVPP
RpDuoX	131	QVVVSSEVMA	//	262	LLGDPRSNQNPALL	//	355	AFRFGHTLIPP

property of dityrosine formed by oxidative protein cross-linking (30, 31). To confirm that this difference in fluorescence was due to a distinct dityrosine content, we subjected eggshells from silenced or control eggs to acid hydrolysis. The fluorescence excitation wavelength spectrum obtained from dsMal sample was subtracted from the dsDuox sample, showing a differential spectrum close to that of standard dityrosine (Fig. 4C).

The role of RpDuox in generating H₂O₂ during chorion formation was further confirmed by reversing the effect of RpDuox silencing by manipulating H₂O₂ levels in the insect, either by injecting H₂O₂ or by injecting dsRNA for catalase together with dsRNA for Duox (Fig. 5). Injection of H₂O₂ did not affect oviposition or the eclosion rate of eggs laid by control females but was able to partially reverse the detrimental effects of RpDuox silencing. Knockdown of catalase, conversely, had a marked effect on oviposition that was partially rescued by simultaneously silencing RpDuox. Interestingly, although silencing either RpDuox or catalase resulted in a pronounced reduction in eclosion, simultaneously silencing both genes resulted in attenuation of this phenotype (Fig. 5).

DISCUSSION

Recently, several research groups have looked for biological functions of Duox in insects. In *D. melanogaster* and *A. aegypti*, Duox has an immunological role in producing H₂O₂ that controls the levels of intestinal microbiota (32, 33). In *A. gambiae*, Duox appears to cross-link peritrophic layer proteins by forming dityrosine bonds on the luminal surface of midgut epithelial cells, a process that limits the diffusion and activity of immune elicitors derived from microbiota (13). In *D. melanogaster*, Duox plays an essential role in the stabilization of the wing cuticle structure by producing the H₂O₂ required during sclerotization, melanization, and tyrosine cross-linking (32). Several studies performed with *D. melanogaster* and *A. aegypti* (2, 5, 6, 33, 34) have demonstrated the importance of chorion protein cross-linking through the oxidation of tyrosine residues and formation of dityrosine bonds during insolubilization of the eggshell protein moiety. In the present work, we show that a Duox enzyme in the *R. prolixus* ovary is the source of the H₂O₂ that is used to cross-link chorion proteins by dityrosine bonding.

The mechanical rigidity of the chorion was recognized a long time ago (1), and it has been attributed to protein cross-linking by peroxidases (2, 4, 5). In the present work, we identified the Duox gene from *R. prolixus* on the basis of genomic and transcriptomic data, characterized its expression profile, and showed that its expression and activity in the ovary increase after a blood meal. In contrast to mosquitoes, in which the hardening of the chorion takes place in the open environment,

after the eggs are laid, in triatomine insects, protein oxidation occurs before oviposition, still in the ovariole. Consistent with this, the highest Duox activities are found in the follicular epithelium during chorion formation. The eggs of Duox-silenced females that hatched on humidity chambers were more fragile than control eggs when pressed with tweezers. Although control eggs broke into pieces when mechanically damaged, dsDuox eggs typically tore. In addition, although control eggshell samples took several hours to solubilize during acid hydrolysis, dsDuox eggshells dissolved completely after only a few minutes of incubation (not shown). As observed originally by Beament (1), this resistance of the eggshell to the harsh conditions used in acid hydrolysis is attributed to the most external layer of the chorion.

If the mechanical strength of the chorion is clearly explained by protein cross-linking, the same does not apply to the protection against water loss. Beament (35) concluded that the wax layer that covers the inner side of the chorion confers water impermeability to the eggs of *R. prolixus*. Moussian *et al.* (36) showed that chitin, the major component of the procuticle of *D. melanogaster*, is not responsible for the protection from dehydration that is conferred by the cuticle. However, Shaik *et al.* (37) suggested that the resistance to water loss presented by the cuticle of *D. melanogaster* larvae is principally due to the dityrosine bonding between cuticular proteins. The results presented here support the conclusion that, similar to the study on insect cuticle, protein cross-linking in the egg chorion is also essential to prevent water loss.

Duox proteins (RpDuox included) are characterized by the presence of a peroxidase-like domain, which is not enzymatically active in some genes described, such as the human Duox1 (10). The catalytic distal histidine (His²⁶¹) and the covalent heme-binding site residues, aspartate (Asp²⁶⁰) and glutamate (Glu⁴⁰⁸), which have been implicated as critical for activity in typical peroxidases (38, 39), are missing in RpDuox (Fig. 6B), similar to what was described for human Duox1 (10). Additionally, we were not able to detect any differences between the peroxidase activities of follicular epithelia from control and Duox-silenced insects using L-tyrosine ethyl ester or 2,2'-azobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrates (data not shown). Some authors, however, support the concept that Duox works as a multiple active site enzyme that generates H₂O₂ while simultaneously catalyzing the formation of dityrosine bonds through its peroxidase domain (9, 10).

H₂O₂ injection and silencing of catalase were both able to partially reverse the reduction in the eclosion rate induced by dsDuox, providing additional support for the role of RpDuox in chorion protein insolubilization and waterproofing. However,

FIGURE 6. **Sequence and structural features of RpDuox.** A, amino acid sequence alignment of insect Duox orthologs. From top to bottom, Duox sequences of *R. prolixus* (RpDuox), *A. pisum* (ApDuox), *D. melanogaster* (DmDuox), and *A. gambiae* (AgDuox) are shown. The consensus sequence is indicated below the alignment. The peroxidase-like domain, EF-hand calcium binding sites, and NOX domain are indicated by the colors yellow, gray, and blue, respectively. The superscripted single, double, and triple bars indicate residues comprising the transmembrane hydrophobic regions (TM1–7), FAD binding sites, and NADPH binding sites, respectively. The sequences were aligned using the Clustalw2 software. Dashes represent gaps introduced to preserve alignment. B, sequence alignment of typical and Duox peroxidase-like domains. From the top to bottom, sequences of *H. sapiens* myeloperoxidase (HsMPO), *H. sapiens* Duox1 (HsDuox1), *C. elegans* Duox1 (CeDuox), and RpDuox are shown. Filled circles indicate the distal and proximal histidines (His²⁶¹ and His⁵⁰², respectively), catalytic arginine (Arg⁴⁰⁵), and covalent heme-binding site residues aspartate (Asp²⁶⁰) and glutamate (Glu⁴⁰⁸), which are critical for the activity of the typical peroxidase HsMPO. Accession numbers are: RpDuox, RPTMP03545; ApDuox, XP_001951113; DmDuox, NP_608715; AgDuox, XP_319115.4; HsMPO, NP_000241.1; HsDuox, gb AAI14939.1; CeDuox, gb AAF71303.1.

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catalase silencing alone severely reduced the number of eggs that were formed, an effect that was also partially reverted by simultaneously silencing *RpDuox*. This effect of dsCat on oogenesis revealed an unanticipated role of redox balance in the regulation of vitellogenesis, suggesting that the fine-tuning of reactive oxygen species levels has an essential role in the regulation of egg maturation, a possibility that deserves further investigation. A role for a Duox enzyme has also been reported in sea urchin eggs, where it acts as a source of H_2O_2 that supports protein cross-linking through dityrosine formation during egg cortex transformation. This process, which starts at the moment of sperm fusion, alters extracellular matrix characteristics, which prevents polyspermy (40) and confers resistance against mechanical distortion and chemical attack, allowing the formation of an appropriate environment for embryo development (41). Because they are aquatic organisms, sea urchins do not have waterproofed eggs. The recognition that these two types of egg extracellular layers, the insect chorion and the sea urchin fertilization barrier, also share the same mechanism of formation leads us to speculate that they may have the same evolutionary origin despite the large phylogenetic distance. If true, this hypothesis would imply an ancestral role for Duox in reproduction.

In summary, the present study shows for the first time that in *R. prolixus*, Duox is the enzyme that generates H_2O_2 in the follicular epithelium and supports chorion peroxidase activity during the hardening of eggshell proteins, which is essential in the acquisition of resistance to water loss.

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