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Catalase is a Determinant of the Colonization and Transovarial Transmission of *Rickettsia parkeri* in the Gulf Coast Tick *Amblyomma maculatum*

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Catalase is a determinant of the colonization and transovarial transmission of *Rickettsia parkeri* in the Gulf Coast tick *Amblyomma maculatum*

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Abstract

The Gulf Coast tick (*Amblyomma maculatum*) has evolved as the competent vector of the spotted-fever group rickettsia, *Rickettsia parkeri*. In this study, the functional role of catalase, an enzyme responsible for the degradation of toxic hydrogen peroxide, in the colonization of the tick vector by *R. parkeri* and transovarial transmission of this pathogen to the next tick generation, was investigated. Catalase gene (*CAT*) expression in midgut, salivary glands, and ovarian tissues exhibited a 2–11-fold increase in transcription level upon *R. parkeri* infection. Depletion of *CAT* transcripts using an RNA-interference approach significantly reduced *R. parkeri* infection levels in midgut and salivary gland tissues by 53%–63%. The role of *CAT* in transovarial transmission of *R. parkeri* was confirmed by simultaneously blocking the transcript and the enzyme by injecting dsRNA-*CAT* and a catalase inhibitor (3-amino-1,2,4-triazole) into gravid females. Simultaneous inhibition of the *CAT* transcript and the enzyme significantly reduced the egg conversion ratio with a 44% reduction of *R. parkeri* transovarial transmission. These data suggest that catalase is required for rickettsial colonization of the tick vector and transovarial transmission to the next generation.

Keywords

Rickettsia parkeri; tick; *Amblyomma maculatum*; catalase; transovarial transmission; vector competence; antioxidants

Introduction

The recent increase in tick-borne diseases poses a significant threat to public health in the absence of preventive measures. Ticks take blood meals of a spectacular size, up to 100 times their unfed weight. While the fundamental mechanisms by which tick-borne pathogens dodge mammalian immune systems have been extensively examined, very little information is available about how these pathogens promote their survival in tick vectors and are passed on to next generation via eggs (transovarial transmission). A dynamic, multi-directional set of interactions between ticks, hosts, and transmitted pathogens occurs in both

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tick, and host environments. These interactions can be regarded as a continuous "*bellum omnium contra omnes*" or war of all against all (Chmelař et al., 2016). The Gulf Coast tick, *Amblyomma maculatum*, has recently gained an increased attention due to its established role as a competent vector of *Rickettsia parkeri*, an emerging rickettsial disease of public-health significance (Paddock and Goddard, 2015; Teel et al., 2010).

Vector competence is a multifactorial process and involves multiple gene networks in multiple organs. Vector colonization is defined as the acquisition, survival, multiplication, and trafficking of a pathogen by a vector. Understanding the functional consequence of pathogen colonization within the tick vector is fundamental to the development of new paradigms for the control and prevention of ticks and tick-transmitted pathogens.

The life cycles of tick-borne pathogens expose them to oxidative stress as ticks become engorged with blood and, for transovarial and trans-stadially transmitted pathogens like *Rickettsia*, to hatching and molting stresses. Ticks must possess physiological mechanisms to maintain redox homeostasis to survive. Their antioxidant network, comprising superoxide dismutase (SOD), catalase, and many selenoproteins, confers resistance to oxidative damage during hematophagy (Adamson et al., 2014, 2013; Budachetri and Karim, 2015; Kumar et al., 2016). Hydrogen peroxides are generated in the cells mainly through dismutation of the superoxide radical formed during cellular respiration and also by a range of oxidase enzymes, including glycolate and monoamine oxidases and the peroxisomal pathway of β-oxidation of fatty acids (Halliwell et al., 2000). Hydrogen peroxide is very soluble in water and readily crosses cell membranes. Although it is regarded as cytotoxic, it is widespread throughout the body and is considered a second messenger in the nuclear factor kappa B (NF-kB) pathway, which regulates many biological functions (Schreck et al., 1991), including the anti-inflammatory response to rickettsial infections (Rydkina et al., 2005). Catalase reduces the excessively generated hydrogen peroxide during physiological or pathological conditions, and it has been reported to extend life span of flies as well (Orr and Sohal, 1994).

The characterization of pathogen-induced gene expression within the tick vector is beginning pathogen colonization and vector competence are poorly understood and urgently need to be addressed. Additionally, the functional role of tick antioxidants in vector competence and colonization is virtually unknown. Specifically, there is a gap in our knowledge of the functional roles played by tick antioxidants in the life cycle of *R. parkeri* bacteria within the tick *A. maculatum*. This study extends earlier catalase work (Kumar et al., 2016) and provides evidence that *R. parkeri* modulate tick catalase conferring survival advantages during rickettsial infection within the tick, and during transovarial transmission.

Results and Discussion

The functional role of tick catalase was investigated in ticks colonized *R. parkeri* and during transovarial transmission to the next generation by depleting *CAT* at the transcript level using an RNAi assay and/or the *CAT* inhibitor, 3-amino-1,2,4-triazole. *R. parkeri*–infected ticks were used to elucidate the role of *CAT* in rickettsial colonization within tick organs and its transovarial transmission. The establishment of spotted-fever group rickettsial agents

within the tick vector and its tissues has been little studied. Mammalian cells have been reported to form superoxide radicals to defend invading rickettsial infections (Santucci et al., 1992). Later, SOD from the host cell but not from the pathogen, restores redox balance, establishing the infection (Santucci et al., 1992). Intriguingly, tick-borne pathogens including *Borrelia burgdorferi* and *Anaplasma phagocytophilum* up-regulate different set of genes in tick vector and their mammalian hosts (Iyer et al., 2015; Villar et al., 2015). Therefore, it is highly unlikely that rickettsial infection within the tick host and mammalian host will follow the same gene expression, and we hypothesized that *R. parkeri* differentially regulate tick antioxidant genes to defend against oxidants. The ability of pathogens (e.g. rickettsia and ehrlichia) to down-regulate the mammalian host immune response and to manipulate host cells is important for their establishment inside eukaryotic cells (Rikihisa, 2010; Sahni et al., 2013; Walker et al., 2003). In *R. parkeri*–infected *A. maculatum*, transcriptional expression of *CAT* was significantly upregulated 11-fold in salivary glands ($p<0.05$) (Fig. 1a), while expression increased 1.5- and 2-fold, respectively, in midgut and ovarian tissues (Fig. 1a). A highly upregulated *CAT* transcript level in *R. parkeri*–infected tick salivary glands suggested tissue specificity of tick-pathogen interactions and a potential role in *R. parkeri* survival and transmission in physiologically active salivary glands before transmission to the vertebrate host (Ayllón et al., 2015; Macaluso et al., 2003). To combat oxidative injury during establishment of rickettsial infections, tick cells possibly upregulated *CAT* to provide redox balance as it correlate with previous findings from an *in vitro* mammalian model (Eremeeva and Silverman, 1998). Other important antioxidants upregulated during infections in mammalian cells include SOD, glutathione peroxidases and heme oxygenases (Rydkina et al., 2004, 2002). These antioxidants were also reported in tick-pathogen interaction studies (Crispell et al., 2016; Narasimhan et al., 2007). Rickettsial gene expression was not examined in the present study, but *Anaplasma phagocytophilum*, another tick borne intracellular pathogen was reported to significantly upregulate stress response genes and surface proteins within the tick vector (Villar et al., 2015).

We achieved 99.8% knockdown of *CAT* transcripts with the RNAi assay in both of the tick organs tested (Fig. 1b). The transcriptional activity of selected antioxidants was evaluated in *CAT* knockdown tick tissues; *Cu/Zn-SOD* (5–21-fold) and *TrxR* (3–3.5-fold) were significantly upregulated in tick midgut and salivary gland tissues ($p<0.05$) (Fig. 1b). *SelM* (3.4-fold) only in gut tissues and *GSHR* (2.7-fold) only in salivary glands were upregulated in *CAT* depleted tick salivary glands (Fig. 1b). Glutathione reductase (*GSHR*) and thioredoxin reductase (*TrxR*) reduce disulfide bonds and catalyze hydrogen peroxide reduction (Zhong et al., 2000), probably compensating for the depletion of *CAT*. Selective activation of glutathione- and superoxide-quenching systems are important defense mechanisms against oxidative damage caused by rickettsial colonization and are essential for mammalian cell survival (Eremeeva and Silverman, 1998; Hong et al., 1998). Similar mechanisms may exist within the tick vector for pathogen survival, and replication. Salivary peroxidase (*Salp25D*), selenoprotein O (*SelO*) and cytoplasmic superoxide dismutase (*Mn-SOD*) were unchanged with *CAT* knockdowns (Fig. 1b). Importantly, there was a significant 53% reduction in *R. parkeri* infection level as quantified by qPCR assay in midgut tissues ($t=3.6$, df=6, $p=0.014$) and about 62% reduction in salivary glands ($t=3.27$, df=5, $p=0.016$) (Fig. 1c). Rickettsial replication in the tick tissues was probably affected by the elevated

oxidative stress caused by *CAT* depletion and insufficient compensation from other antioxidants (Fig. 1b). The rickettsial antioxidants and stress response genes that were reported to significantly increase during tick infections probably ameliorated the adverse impact of *CAT* knockdown, which may explain why we did not observe complete clearance of *R. parkeri* (Villar et al., 2015).

Both dsCAT+AT-injected and 1×PBS-injected gravid females were allowed to lay their eggs throughout the laying period of 25–30 days (Drummond et al., 1970). Tick fecundity was severely impaired in catalase-knockdown gravid females compared with control ticks, as reported earlier in *R. parkeri* free ticks (Kumar et al., 2016) (Fig. 2 d–g). The egg conversion ratio, which measures oviposition efficiency, was significantly reduced in *CAT*-depleted gravid females (p=0.035) (Fig. 2 g). Reduced oviposition with the silencing of catalase has been reported in blood-feeding ticks and mosquitoes (DeJong et al., 2007; Kumar et al., 2016). Moreover, in this study we showed the role of tick *CAT* in transovarial transmission of *R. parkeri* (Fig. 2 h). *R. parkeri* was quantified by qPCR assay in tick egg cohorts of dsCAT+AT-injected and 1×PBS-injected gravid females, which showed significant reduction of the *R. parkeri* load by about 44% (t=2.66, df=5, p=0.045) with silencing of tick catalase (Fig. 2h). The transcriptional expressions of *CAT* along with antioxidants in tick egg cohorts from the *CAT* silenced gravid females were either depleted (*TrxR* and *SelO*) or remained similar to the egg cohorts from 1×PBS injected gravid females, which suggests no silencing impact in eggs (Fig. 2i).

Tick catalase, an antioxidant that detoxifies hydrogen peroxide synthesized during cellular respiration and many oxidase reactions during hematophagy, is known to balance redox and tick fecundity (DeJong et al., 2007; Kumar et al., 2016). The RNAi assay coupled with the catalase inhibitor assay showed reduced *R. parkeri* survival in tick tissues and reduced transovarial transmissions (Fig 1, 2). The rickettsial antioxidant and stress response genes were not studied but are determining factors in pathogen strategies for evading the tick's innate immune response and host manipulations (Mansueto et al., 2012; Pornwiroon et al., 2015). Tick antioxidants, in combination with pathogen stress response genes, when silenced together may help to eliminate *R. parkeri* from *A. maculatum*. Interestingly, an infection level of *R. parkeri* <40% has been reported in field collected A. maculatum, but once the female tick is infected it maintains infection level across the life cycle and many generations (Budachetri et al., 2014; Wright et al., 2015). The intricate symbiosis between tick and rickettsia, which is maintained throughout the tick life cycle, will be revealed only by employing differentially expressed genes from both tick and pathogen sides.

Experimental Procedures

Rickettsia parkeri–infected ticks

The spotted-fever group rickettsia, *R. parkeri*–infected ticks were maintained in the lab as described and used earlier (Adamson et al., 2013; Budachetri et al., 2014; Crispell et al., 2016). All studies were conducted using an approved protocol from the University of Southern Mississippi's Institutional Animal Care and Use Committee (IACUC).

Rickettsial colonization: *CAT* silencing in Rp+ infected unfed females

The preparation of dsRNA, tick injections, tick handling and tick tissue isolations were performed as described in earlier publications (Kumar et al., 2016). The dsRNA for CAT and dsLacZ were injected into each of 20 *R. parkeri*–infected adult females and allowed to feed on sheep. The ticks were pulled from sheep on day 5 to assess silencing of *CAT* and the responses of other tick antioxidants. The ticks were then dissected and the midguts and salivary glands were isolated from each tick and placed separately in RNAlater and placed in −80°C until further use.

Transovarial transmission of *R. parkeri*: *CAT* silencing in Rp+ gravid females

Ten engorged Rp+ female ticks were injected with either a cocktail of dsCAT (1 μg/μL) and 3-amino-1,2,4-triazole (AT, 20 mM), or 1×PBS, to observe the impact of *CAT* knockdown on the transovarial transmission of *R. parkeri*. The egg conversion ratio (tick egg mass after detachment from host/gravid female mass) was observed after completion of tick oviposition over 25 days from three PBS injected and 5 dsCAT+AT injected ticks. The 20 mg of eggs obtained from each tick was used to isolate total RNA and analyzed for quantification of *R. parkeri.*

Total RNA isolation, cDNA synthesis, and transcriptional expression

Total RNAs were extracted from dissected tick tissues (tick organs and eggs) as described previously (Crispell et al., 2016). All primers (Table 1) and thermal cyclic parameters used in this study were as published previously (Kumar et al., 2016). *CAT* expression in *R. parkeri*–infected tick organs was compared with that in uninfected ticks to confirm silencing of *CAT* in the dsRNAi assay. Compensatory transcriptional activities of selected tick antioxidants in *CAT* knockdown tissues were determined by qRT-PCR assay, as described earlier (Kumar et al., 2016).

Quantification of *Rickettsia parkeri* in tick tissues

The *R. parkeri* load (Rp load) in tick tissues was estimated by quantifying *R. parkeri* copies using rickettsial outer membrane protein B (*rompB*) per tick *GAPDH* copy in tick tissues. *rompB* was amplified from *R. parkeri*–infected tick tissues using specific primers described earlier (Table 1) (Jiang et al., 2012); tick *GAPDH* was amplified in a thermal cycler and PCR products were purified, sequenced, and verified prior to further assays. Two standard curves of tick *rompB* and *GAPDH* genes were determined based on serially diluted PCR products. The serial dilutions of 10^8 to 10^1 copies of each gene were amplified and tick tissue cDNAs were prepared in 20 μL of qRT-PCR reaction medium, which consisted of 250 nM of each of primer, and SYBR Green Master Mix (BioRad Inc. CA, USA). Reaction mixtures were subjected to 95°C for 10 min, followed by 35 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s using the CFX96 Real Time System (BioRad Inc.). Two standard curves were prepared based on the corresponding Ct values of known standard dilutions for the two genes. The copy numbers for *R. parkeri* and tick *GAPDH* were estimated based on standard curves.

Data Analysis

Data are presented as means ± SEM and significance differences between two groups were estimated by Student's *t*-test or Mann-Whiteny-Wilcoxon test in Graph Pad software (Graph pad Prism 6.05, La Jolla, CA). Transcriptional expressions were analyzed by the ΔΔCt method in Bio-Rad Software (Bio-Rad CFX manager V 3.1) and two-fold differences with *p*-values <0.05 were considered significant. All qRT-PCR assays were performed on at least three samples per group, with technical duplicates.

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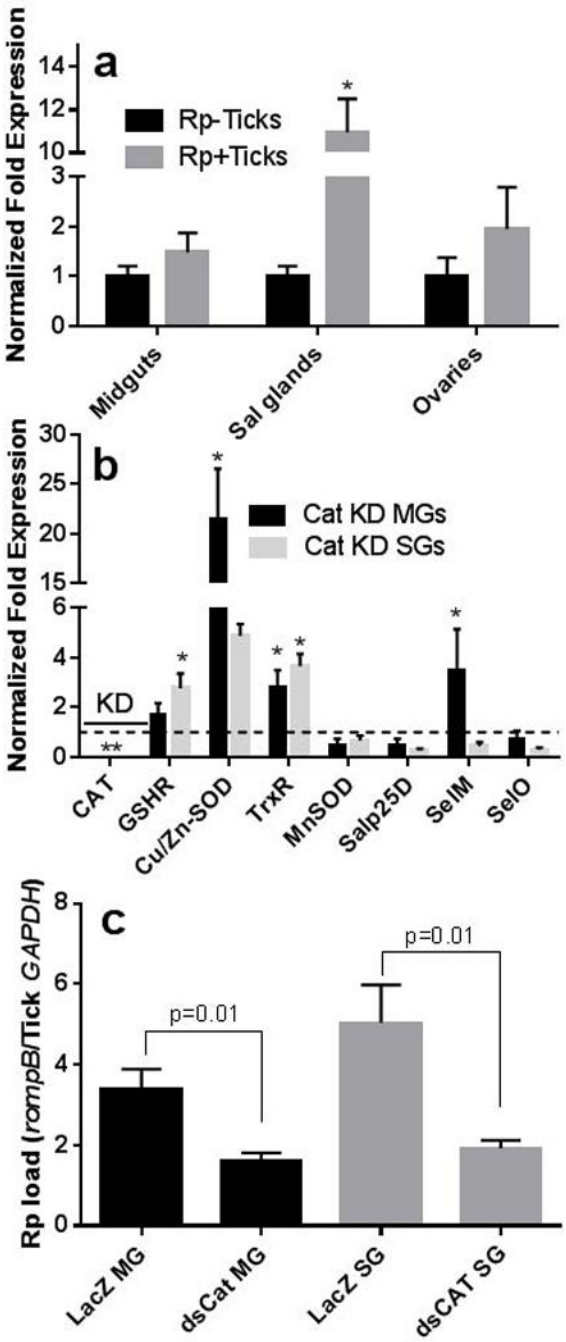

Figure 1.

Role of tick catalase (CAT) in Rickettsia parkeri colonization in Amblyomma maculatum. (a) The transcriptional expressions of tick catalase was estimated in R. parkeri-infected tick (Rp (+) ticks) organs and compared with that in R. parkeri-free (Rp (−) ticks) organs. (b) RNAi assay was used to silence tick CAT in R. parkeri infected ticks and transcriptional expressions of GSHR, Cu/Zn-SOD, MnSOD, TrxR, SelM, SelO, and Salp25D were estimated with CAT knockdown. The expression level of each target gene in control tick organs was assigned a value of 1. The transcriptional expressions were normalized against

tick GAPDH as a reference gene. Symbol (*) denotes statistical significance at p-value <0.05. (c) The R. parkeri load was estimated by qPCR assay in LacZ- (irrelevant control) and CAT-injected tick tissues. Rp (+) ticks, R. parkeri infected tick; Rp (−) ticks, R. parkeri free ticks; MG, midgut; SG, salivary glands; GSHR, glutathione reductase; SOD, superoxide dismutase; TrxR, thioredoxin reductase; SalP25D, tick salivary gland peroxidase homologue; SelM, selenoprotein M; SelO, selenoprotein O; GAPDH, glyceraldehyde 3-phosphate.

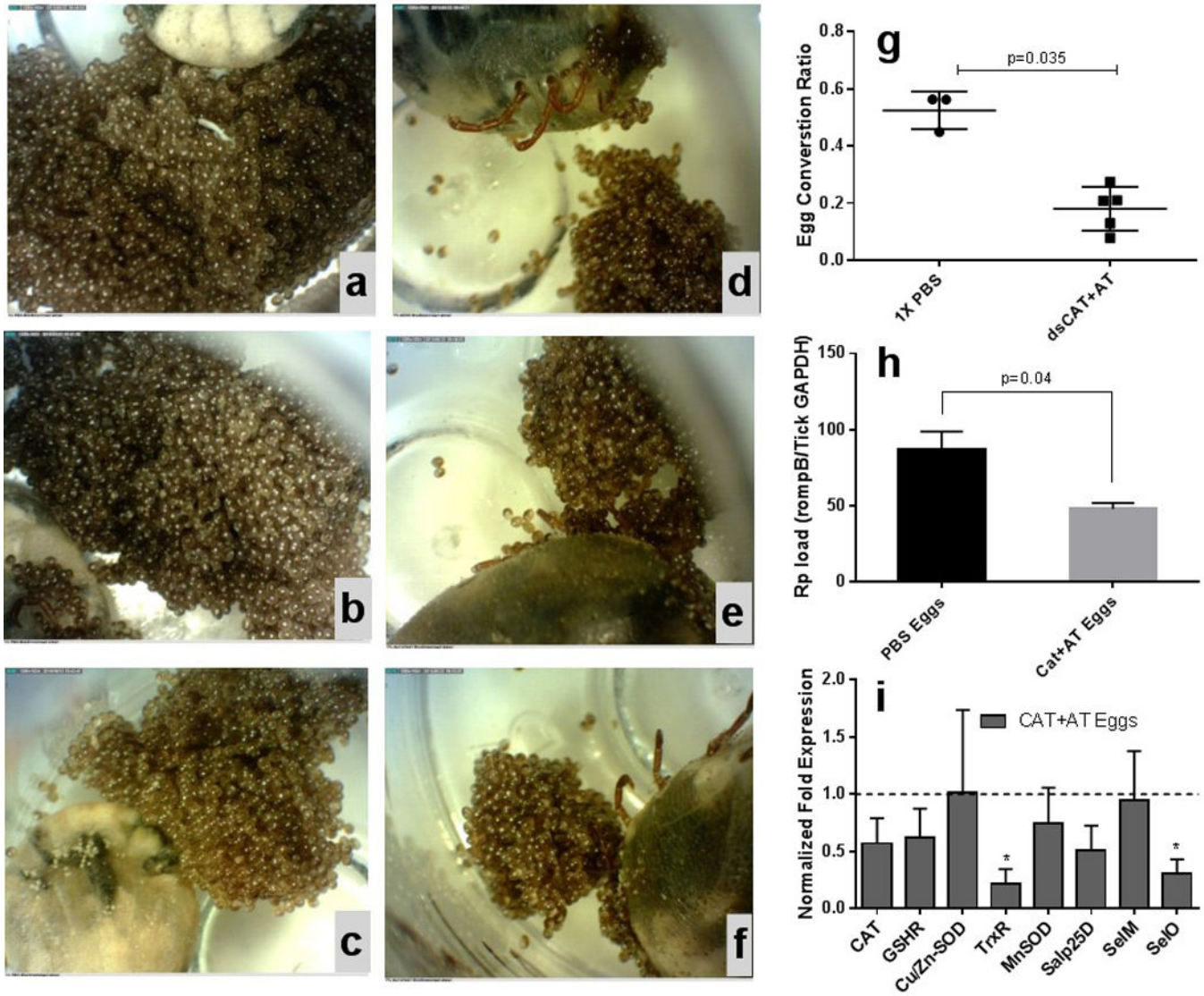

Figure 2.

Tick catalase (CAT) plays role in transovarial transmission of Rickettsia parkeri. (a–c) Eggs from 1×PBS and (d–f) 1 μL of cocktail of (1 μg/μL of dsCAT and 20 mM of 3-amino-1,2,4-triazole (AT) injected R. parkeri-infected gravid females. (g) Egg conversion ratio (tick egg mass/tick weight of gravid female at engorged stage). (h) R. parkeri load in tick egg masses after the completion of the oviposition period of 25 days in A. maculatum. (i) Relative transcriptional levels of tick antioxidants in eggs from gravid females injected with 1×PBS or with dsCAT+AT with tick GAPDH as the reference. The level of expression of target genes in 1×PBS was assigned a value of 1. GSHR, glutathione reductase; SOD, superoxide dismutase; TrxR, thioredoxin reductase; SalP25D, tick salivary gland peroxidase homologue; SelM, selenoprotein M; SelO, selenoprotein O; GAPDH, glyceraldehyde 3-phosphate.

Table 1

Oligonucleotides used in this study.

Gene	GenBank ID	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Size (bp)
Catalase	JO843741	AAAGGACGTCGACATGTTCTGGGA	ACTTGCAGTAGACTGCCTCGTTGT	173
GSHR	JO844062	ACCTGACCAAGAGCAACGTTGAGA	ATCGCTTGTGATGCCAAACTCTGC	170
SelM	JO842653	GCTGTACGATGAGAATGGAGAG	AGCCAGGTGCTCAAACAA	94
SelO	KC989561	AAGCTCGGCCTTGTGAAGAGAGAA	TACAGCACGACAAGAGCTTGGACA	190
Cu-Zn SOD	JO844140	GGAACCGAAGACAGCAAGAA	GAGAAGAGGCCGATGACAAA	143
Mn- SOD	JO843979	GCATCTACTGGAC AAACCTCTC	GCAGACATCAGGCCTTTGA	115
TrxR	JO843723	TGTGACTACACCAACGTGCCTACA	AGTAGCCTGCATCCGTTCCTCTTT	175
rOmpB	AF123717	CAAATGTTGCAGTTCCTCTAAATG	AAAACAAACCGTTAAAACTACCG	96
GPx/Salp25D	JO843645	TGCCGCGCTGTCTTTATTATTGGC	AGTTGCACGGAGAACCTCATCGAA	102
GAPDH	JO842341	CACCCATCACAAACATGGGTGCAT	TTTCAGGAAATGAAGCCTGCCAGC	175