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Amblyomma maculatum SECIS binding protein 2 and putative selenoprotein P are indispensable for pathogen replication and tick fecundity

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Abstract

Selenium, a vital trace element, is incorporated into selenoproteins to produce selenocysteine. Our previous studies have revealed an adaptive co-evolutionary process that has enabled the spotted fever-causing tick-borne pathogen *Rickettsia parkeri* to survive by manipulating an antioxidant defense system associated with selenium, which includes a full set of selenoproteins and other antioxidants in ticks. Here, we conducted a systemic investigation of SECIS binding protein 2 (SBP2) and putative selenoprotein P (SELENOP) by transcript silencing in adult female Gulf-coast ticks (*Amblyomma maculatum*). Knockdown of the *SBP2* and *SELENOP* genes depleted the respective transcript levels of these tick selenogenes, and caused differential regulation of other antioxidants. Importantly, the selenium level in the immature and mature tick stages increased significantly after a blood meal, but the selenium level decreased in ticks after the *SBP2* and *SELENOP* knockdowns. Moreover, the *SBP2* knockdown significantly impaired both transovarial transmission of R. parkeri to tick eggs and egg hatching. Overall, our data offer new insight into the relationship between the SBP2 selenoprotein synthesis gene and the putative tick *SELENOP* gene. It also augments our understanding of selenoprotein synthesis, selenium maintenance and utilization, and bacterial colonization of a tick vector.

Keywords

Amblyomma maculatum; Selenium; Selenoproteins; *Rickettsia parkeri*; RNA interference

1. Introduction

Tick blood feeding generates toxic levels of reactive oxygen species (ROS) that can damage lipids, proteins, and DNA, thus promoting mutation, cellular dysfunction, and cell death. Therefore, to successfully feed and survive, ticks must somehow prevent the detrimental and promote the beneficial aspects of ROS release, which suggests that precise regulatory strategies must exist for maintaining appropriate ROS levels both within the tick and

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possibly at the tick—host interface. Our previous studies have revealed an adaptive co-evolutionary process that enables the survival of the tick-borne pathogen *Rickettsia parkeri* by manipulating an antioxidant defense system associated with selenium, which includes a full set of selenoproteins and other antioxidants (Adamson et al., 2014, 2013; Budachetri et al., 2017; Budachetri and Karim, 2015; Crispell et al., 2016; Karim et al., 2011; Karim and Ribeiro, 2015; Kumar et al., 2016). ROS generation is one of the first lines of host defense against invading microbes (Ha et al., 2005). Selenoproteins exhibit diverse biological functions such as detoxification of peroxides, regeneration of reduced thioredoxin, and reduction of oxidized methionine residues by oxidation of the selenium (Se^{-}) active site (Gromer et al., 2005; Reeves and Hoffmann, 2009). The number of selenoproteins present in an organism varies widely: 10–57 in algae, 30–37 in fish, and 23–25 in mammals (Lobanov et al., 2009; Mariotti et al., 2012). However, selenoproteins are reduced in number or lost altogether in most arthropods. Instead, some insect species possess cysteine-containing homologs or may lack selenoproteins altogether, despite them being essential in mammalian systems (Chapple and Guigó, 2008; Lobanov et al., 2008; Shchedrina et al., 2010a). Recent studies have shown that the selenocysteine incorporation machinery is absent in at least five insect species (*Tribolium castaneum, Bombyx mori, Drosophila willistoni, Apis mellifera,* and *Nasonia vitripennis*) (Hirosawa-Takamori et al., 2004; Shchedrina et al., 2011). Instead, they possess cysteine-containing homologs and, where it is present, the selenoproteome is reduced to only one to three selenoproteins, as is the case forD. *melanogaster* and *Anopheles gambiae* (Hirosawa-Takamori et al., 2004; Shchedrina et al., 2011). The evolutionary reduction of selenoprotein use may be linked to the significant changes that have occurred in insect antioxidant systems. Interestingly, the role of selenoproteins in *Drosophila* does not appear to be critical for either lifespan or oxidative stress defense (Hirosawa-Takamori et al., 2004). In contrast, despite the lack of published research, there is evidence that the tick selenoproteome and antioxidant enzymes may play critical roles in detoxifying ROS and maintaining both vector microbiota and *R. parkeri* colonization (Adamson et al., 2013; Budachetri et al., 2017; Budachetri and Karim, 2015; Crispell et al., 2016; Kumar et al., 2016).

Selenium is an essential trace element required for the proper functioning of organisms. It participates in protecting cells against excess H_2O_2, in heavy metal detoxification, and in regulating the immune and reproductive systems (Kieliszek and Błażejak, 2016). With its known biological activity, selenium is important for human and animal nutrition, and for health and immune system functioning (Kieliszek and Błażejak, 2013; Scott, 1973). Selenium deficiency causes the degeneration of many organs and tissues from changes in the biological processes in which it participates via decreased selenoprotein expression (Pedrero and Madrid, 2009). Moderate deficiencies may also increase the risk of infertility, prostate cancer, nephropathy, and neurological diseases (Kieliszek and Błażejak, 2016). Conversely, the chronic toxicity caused by excess selenium in living organisms leads to selenosis, as manifested by hair loss, infertility, fragility or other changes in the fingernails or hooves, gastrointestinal disturbances, skin rashes, an unpleasant garlic-like odor in exhaled air (from dimethylselenide), and nervous system disorders (Li et al., 2012; Nazemi et al., 2012). Selenium contributes to normal cell growth and plays an important role in modulating the action of transcription factors and cell signaling systems. The biological roles for selenium

include the prevention of infertility, diabetes, cancer, and cardiovascular diseases (Hendrickx et al., 2013).

The specific functions of selenoproteins involve thiol-based redox signaling, control of reduced cysteine (Cys) residues in cytosolic and mitochondrial proteins, removal of hydrogen peroxide, repair of oxidatively damaged proteins, control of cytoskeleton and actin assembly, selenoprotein synthesis, selenium transport, protein folding, and endoplasmic reticulum (ER)-asso-ciated degradation, among others (Labunskyy et al., 2014). Selenocysteine (Sec), the 21st naturally occurring amino acid, is inserted into nascent polypeptides at UGA codons. Incorporation of Sec instead of Cys often results in higher enzyme activity, providing a competitive advantage to the organisms that utilize it (Kim and Gladyshev, 2005). Sec incorporation requires the presence of complex machinery comprising Sec-tRNA elongation factor (eEF-sec), Sec insertion sequence (SECIS)-binding protein 2 (SBP2), selenophosphate synthetase 2, phosphoseryl-tRNA kinase, SECp43, Sec synthase, and a cis-acting stem-loop structure known as the SECIS element in the 3′-untranslated region (Hatfield et al., 2006). At both the cellular and physiological levels, the functional role of selenium has yet to be investigated in blood-sucking arthropods such as ixodid ticks. Tick-borne pathogens manipulate the gene expression of their vector hosts to ensure their survival and replication and their onwards transmission (vector competence) to mammalian hosts. Very little is known about the relationship between redox balance and the growth and viability of intracellular bacteria. In mammals, invading bacterial pathogens infect neutrophils and professional phagocytes and stimulate the assembly of a nicotinamide adenine dinucleotide phosphate oxidase complex in the vacuole that destroys the bacteria (Hong et al., 1998; Rada and Leto, 2008). *R. rickettsii*, a close relative of *R. parkeri*, generates ROS as a means of pathogenesis in human endothelial cells (Hong et al., 1998). In contrast, *Anaplasma phagocytophilum*, another Rickettsiales member, scavenges ROS extracellularly by secreting SOD1 (Dumler et al., 2005). Studies using other vector-borne disease models have shown that the initial transmission stage of a pathogen by an arthropod vector is influenced by gene expression changes in both the vectors and the pathogens (Hovius et al., 2007).

The redox property of a selenoprotein depends on the selenium atom in the selenocysteine residue. Selenium is unable to form many pi bond types, but its ease of reducing organic and inorganic hydrogen peroxides without oxidative inactivation makes selenoproteins unique antioxidants (Reich and Hondal, 2016). Selenoprotein P (SELENOP) is a multiple selenocysteine (Sec, U)-containing selenoprotein, and most selenium (>50% of the body's total) in human plasma is associated with this protein. Plasma SELENOP acts as a selenium level indicator in the body and is considered to be involved in selenium storage and transport across the body (Richardson, 2005). In this study, we used RNAi to silence two important selenoprotein synthesis machinery genes, SBP2 and the putative tick *SELENOP*, and characterized their roles in tick physiology, selenium status, pathogen growth, and transovarial transmission.

2. Experimental procedures

2.1. Ticks and tick tissue isolation

R. parkeri infected (Rp+) and *R. parkeri* free (Rp-) Gulf coast tick (*A. maculatum*) colonies were maintained at the University of Southern Mississippi (USA) according to established methods (Patrick and Hair, 1975). Ticks were kept at room temperature under approximately 90% relative humidity and a photoperiod of 14 h light/10 h dark before infestation on sheep. Immature ticks were blood fed on hamsters, whereas adult ticks were blood fed on sheep. Immature and mature tick stages were allowed to replete or were removed 3–10 days post-infestation (dpi) of their hosts, depending on the experimental protocol. The Institutional Animal Care and Use Committee at the University of Southern Mississippi approved all the protocols (#10042001 & 15101501) before the experiments commenced.

2.2. Bioinformatics analyses

The *SBP2* (GenBank:MF115980) and *SELENOP* (GenBank: MF115978) coding sequences were obtained from the *A. maculatum* sialotranscriptome (Karim et al., 2011). Both nucleotide sequences were conceptually translated into their amino acid sequences and multiply aligned with their orthologous protein sequences using ClustalX2. For SBP2, the Homo sapiens, human (Q96T21), *Equus caballus*, horse (F6V110), *Danio rerio*, Zebra fish (F1R3N8) and *Ixodes scapularis*, blacklegged tick (B7Q610) sequences obtained from uniprot (www.uniprot.org) (Larkin et al., 2007; Thompson et al., 2002) were graphically represented in Jalview after multiple sequence alignment against tick SBP2 (Jones et al., 1992). The phylogenetic tree was built using MEGA6 software (Tamura et al., 2013) using additional SBP2 sequences from *Oryctolagus cuniculus*, European rabbit (G1T4E3), *Rattus norvegicus*, rat (Q9QX72), *Mus musculus*, house mouse (NP_083555.1), *Clupea harengus*, Atlantic herring (XP_012670383.1), Poeciliopsis *prolifica*, blackstripe live-bearer fish (A0A0S7GUM1), *I. scapularis*, blacklegged tick (ISCW021662 or B7Q610) for phylogenetic insight.

Amblyomma maculatum SELENOP was aligned with SELENOP from *I. scapularis* (B7Q2F3), *I. ricinus* (V5GWR4), *Rhipicephalus appendiculatus* (hereafter referred to as *Rh. appendiculatus*) (A0A131YE48), *Homo sapiens* (P49908), *R. norvegicus* (P25236), and *M. musculus* (P70274), and with two sequences from *Danio rerio* (Q98SV1 and Q98SV0), after which they were visualized in Jalview and MEGA6 software was used to obtain their phylogenetic history (Tamura et al., 2013). The SELENOP-based sequence alignments of the aforementioned organisms were obtained using MEGA6 software after changing all the selenocysteine residues to cysteine (U to C). The sequences showed marked differences between the vertebrates and the ticks, with the three tick species (*R.* appendiculatus, A0A131YE48; *I. scapularis,* B7Q2F3; and *I. ricinus,* V5GWR4) grouping close together in the alignment, except for *Danio rerio* (Q98SV0), which was aligned separately from either group (Fig. S2).

2.3. RNAi assay for tick SBP2 and SELENOP

Double-stranded RNA (dsRNA) synthesis (for *SBP2* and *SELENOP)* and tick manipulations were performed according to the methods described previously (Crispell et al., 2016). The

dsRNAs for SBP2, SELENOP and LacZ (irrelevant control) were diluted to each make a working concentration of 1 μg/μL Groups of 20–25 unfed adult female Rp (+) ticks were microinjected with *SBP2, SELENOP* or *LacZ* using a 27-guage needle. For sample collection, 12 partially fed control ticks were removed on days 5 and 7 post-tick infestation and the remaining ticks allowed to stay attached and blood feed until fully engorged. The feeding success of each female tick was evaluated by three parameters: attachment duration, repletion weight, and the ability to oviposit (Karim et al., 2012). Eggs (20 mg) from the dsRNA-injected ticks were collected from the control and treatment groups (dsLacZ, dsSBP2, and dsSELENOP), stored in RNAlater, and placed at −80 °C. The partially fed ticks removed from each group were dissected to obtain their midguts and salivary gland tissues. All experiments were repeated twice.

2.4. RNA preparation, cDNA synthesis, and quantitative reverse transcriptase PCR (qRT-PCR)

The methods used for RNA extraction from tick tissues (time points, RNAi tissues and eggs), cDNA synthesis and qRT-PCR have been published previously (Bullard et al., 2016). Transcriptional gene expression was normalized against the glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)* gene. All the genes used in this study were first amplified using gene-specific primers (Table S1), and their sequences were confirmed by sequencing before conducting double-stranded (ds) RNA synthesis or before starting the gene expression studies. iTaq™ Universal SYBR® Green Supermix (Bio-Rad Inc., Hercules, CA, USA), 25 ng of cDNA, and 150 nM of gene-specific primers were used in each reaction mixture. The qRT-PCR mixtures were subjected to 10 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C using the CFX96 Real Time System (Bio-Rad Inc., Hercules, CA, USA).

2.5. Immunoblot analysis of tick SBP2 and SELENOP

Individual whole ticks from the no-treatment, dsLacZ, dsSBP2-or dsSELENOP-injected groups were separately processed for protein isolation followed by sonication. All the procedures for protein isolation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting were those reported previously by Browning and Karim (2013) and Budachetri et al. (2014). Nitrocellulose membranes were incubated with mouse anti-selenoprotein P (an anti-human mouse monoclonal antibody, 1:200) in iBind™ apparatus (ThermoFisher Scientific, Waltham, MA, USA) and anti-mouse HRP secondary IgG (1:2000). The same blots were stripped and re-probed with anti-SBP2 1: 750 (iBind™ apparatus), an anti-rat rabbit polyclonal antibody, and the secondary antibody was anti-rabbit HRP IgG (1:10,000). After successful imaging, each blot was re-probed with mAb (GA1R) HRP conjugate (1: 1200; ENZ-ABS276-0050, Enzo life Sciences, Inc., NY, USA). The antigen-antibody complexes were visualized with a horseradish peroxidase-conjugated secondary antibody and SuperSignal chemiluminescent substrate (ThermoFisher Scientific) using the ChemiHi sensitivity method with Chemidox XRS (Bio-Rad, USA).

2.6. Quantifying Francisella-like endosymbionts (FLE), Candidatus Midichloria mitochondrii (CMM) endosymbionts, and R. parkeriloads in ticks

The *R. parkeri* load per tick was determined by quantifying the copy numbers of the rickettsial outer membrane protein B (*ompB*) and *GAPDH* using qRT-PCR, as described earlier (Budachetri et al., 2017; Jiang et al., 2012). The bacterial load in each tick tissue was estimated as described previously (Budachetri and Karim, 2015; Narasimhan et al., 2014). A slightly modified published protocol was used to estimate FLE (Dergousoff and Chilton, 2012) and CMM numbers (Sassera et al., 2006). For each gene standard curves were constructed for quantifying the total bacterial load, the Rp load, the CMM load and the FLE load using the method of Budachetri et al. (2017). Similarly, tick *GAPDH* standard curves corresponding to each quantification protocol were prepared. The copy numbers were calculated using the respective standard curves and the loads represented the number of *GAPDH* copies per tick.

2.7. Lipid peroxidation estimation

The lipid peroxidation assay kit measures the malondialdehyde (MDA) levels formed after peroxidation of polyunsaturated lipids by reactive oxygen species, thereby providing the oxidative stress level in tissues (Crispell et al., 2016). With this kit, lipid peroxidation is determined by estimating the amount of MDA reaction product with thiobarbituric acid. The reaction is measured calorimetrically (wavelength of 532 nm), and peroxidation is proportional to the MDA level in the sample. All the procedures we used followed the manufacturer's recommendations (Sigma-Aldrich, St. Louis, MO, USA).

2.8. Analysis of selenium as a trace element in tick samples

Selenium levels were quantified in tick samples across all the developmental stages including the *SBP2*- and SELENOP-silenced tick samples. We utilized inductively coupled plasma-mass spectroscopy (ICP-MS) to estimate low selenium concentrations (<2 μg/ g) (Kasaikina et al., 2011a; Miksa et al., 2005). Approximately 0.1 g of sample was weighed and placed in a Teflon container with 1 mL ultrapure water and 1 mL ultrapure concentrated HNO_3 (Seastar Baseline, Sidney, British Columbia, Canada). The container was sealed and heated at 110 °C for 8 h. After cooling, 8 mL of ultrapure 0.1% HNO_3 containing 2 parts per billion was added to each sample. The samples were analyzed using a sector field ICP mass spectrometer (ThermoFisher Element XR) operated in high-resolution mode. Isotope scanning included 77Se, 78Se, 82Se, while 115In was used as an internal standard. Calibration was performed using external standards. Generally, no significant difference was noted for the results from the three isotopes. The relative standard deviation of the samples well away from the detection limit (0.005 μg-Se/g) was 14%.

2.9. Data analysis

All data are expressed as mean ± SEM unless otherwise mentioned. Statistical significance between the two experimental groups or their respective controls was determined by the Mann-Whitney rank sum test or *t*-test (P-value, 0.05). Transcriptional expression levels were determined by Bio-Rad software (Bio-Rad CFX MANAGER v.3.1), and expression values were considered significant when the p-value was = 0.05, when compared with the control.

3. Results

3.1. Bioinformatic analyses

A. maculatum SBP2 shared 27–28% sequence identity with the vertebrate, *E. caballus*, and *D. rerio* sequences, and 42–45% with the sequences from the prostriate tick species (i.e., the ixodid ticks like *I. scapularis* and *I. ricinus* V5IFJ7 that have an anal groove in front of the anus). The grey highlight 96 amino acid residues stretch denotes the conserved domain between the vertebrates and tick SBP2 (Fig. S1). The amino acid identity scores in the conserved domains showed that *A. maculatum* shares 54–56% similarity with vertebrates and about 73.96% identity with *I. scapularis*. The SBP2 sequences from each taxonomic class formed their own clades (Fig. S3).

The putative *A. maculatum* SELENOP was found to share 27–28% sequence identity with vertebrate SELENOP from rat, mouse and humans, but less than both SELENOPs from zebrafish (23% identity) (Fig. S2), which is very low compared with the 72% identity scores for human, mouse, and rat. Despite the 92% sequence similarity scores between *I. scapularis* and *I. ricinus* SELENOPs, *Amblyomma* SELENOP shared only 36% sequence identity with both Ixodes SELENOPs. The SELENOP sequence from *Rh. appendiculatus* shares 67% identity with that from *Amblyomma,* but only 36–37% with the *I. scapularis* and *I. ricinus* sequences (Fig. S2).

The multiple sequence alignment for SELENOP is shown in Fig. S2. The sequences enclosed in blue boxes represent a signal peptide indicative of the secretory nature of SELENOP from vertebrates alone. The red boxes highlight the selenocysteine (U) residues, which are selenium-containing amino-acids specific to selenoproteins. On the N-terminal side, up to the poly histidine region of sequences represented by black borders with many histidine residues, only one selenocysteine residue was observed.

3.2. Blood meal and pathogen-induced tick gene expression

The transcriptional activity of *SBP2* became depleted after a blood meal in midgut tissues and salivary glands, except for the midgut tissues at 8 days post infestation (dpi), which had a similar transcriptional level to that of the unfed stage (Fig. 1A). The tran-scriptional activities of *SELENOP* and selenophosphate synthetase (*SEPHS2*) diminished after the blood meal in the slow and fast feeding stages (Fig. 1B–C) whereas in the midgut tissues *SELENOP* was up regulated >3-fold in the slow feeding stage but diminished later (Fig. 1B). Contrastingly, the transcriptional activity of *SEPHS2* was up regulated >40 fold in the midgut tissues of the fast feeding stage (Fig. 1C). The transcriptional expression of *SBP2*, *SELENOP* and *SEPHS2* were estimated in the *R. parkeri*-infected tick tissues to assess the tick response to pathogen infection (Fig. 1D). In both tissue types, SBP2 expression was upregulated significantly during the *R. parkeri* infections, with a 2-fold increase in the midgut tissues ($p < 0.005$) and a 5-fold increase in the salivary glands ($P < 0.005$). Similarly, the presence of a rickettsial infection increased *SELENOP* expression by 2- and 6-fold in the midgut and salivary gland tissues ($P < 0.05$), respectively, whereas the *SEPHS2* transcript level increased ~3-fold in the midgut tissues (Fig. 1D).

3.3. Blood meal and pathogen induced selenium concentrations inticks

We did not observe significant differences in the selenium levels between the Rp+ and Rp- ticks except in eggs ($p = 0.009$), whereas the selenium level increased significantly after a blood meal in all the tick developmental stages, irrespective of the presence of a pathogen infection (Fig. 2A–C). The selenium concentrations increased after the blood meal in both Rp- (about 3-fold) and Rp + larva (about 7-fold) ($p = 0.016$) (Fig. 2A). There were no unfed nymphal tick samples, but in adults after the blood meal both Rp-(2-fold) and Rp+ (1.3-fold) males showed increased selenium concentrations (Fig. 2B). The selenium levels in the blood-fed female ticks increased by 7-fold in the absence of *R. parkeri* ($p = 0.0004$), and 16-fold when infected ($p = 0.0004$), compared with the unfed control (Fig. 2B). The maximum selenium concentration in ticks reached 0.3 μg per g in the adult female ticks. Selenium concentration changes were also observed in *I. scapularis* and *A. americanum* (Fig. 2C). Selenium concentrations in the partially fed *I. scapularis* ticks showed an approximate 7-fold increase, whereas *A. americanum* showed a 5-fold increase (Fig. 2C), compared with the unfed controls.

3.4. SBP2 and SELENOP silencing

3.4.1. Impact on the tick selenoproteome—A significant depletion of SBP2 transcript levels (93%) in midguts and salivary glands (90%) was achieved in the partially blood fed ticks (Fig. 3A). The *SELENOP, SELENOS, SELENOK*, thioredoxin reductase (*TXNRD2*), *SELENON, SELENOT,* and *MSRB1* (*SelX*) transcript levels were depleted in the ticks when *SBP2* was knocked down, while *SEPHS2* and *SELENOO* transcripts were significantly upregulated, and *eEFSec* and *SELENOM* remained unaffected (Fig. 3A). Interestingly, catalase expression levels remained unchanged despite significant upregulation of superoxide dismutase (*Cu/Zn-SOD*) in the *SBP2* knockdown tissues (Fig. 3A).

The *SELENOP* transcript levels were depleted by 96% and 97% in both tick midgut and salivary glands, respectively, upon gene silencing (Fig. 3B). A significant increase in *SEPHS2, SBP2*, and *Cu/ Zn-SOD* transcript levels was observed in the *SELENOP*-silenced midgut tissues (Fig. 3B). *SELENOO* and *TXNRD2* were also significantly upregulated in tick salivary glands (Fig. 3B). *SELENOS* transcripts in both tick tissues, *MRSB1* (*SelX*) in midguts, and *catalase* in salivary glands were all significantly depleted following *SELENOP* knockdown (Fig. 3B).

3.4.2. Immunodetection of SBP2 and SELENOP in the knockdowns—The SELENOP antibody cross-reacted with a tick protein of ≈30 KDa in both the non-treated and irrelevant dsRNA-LacZ-treated ticks, while no cross reactions occurred with the dsSBP2 or dsSELENOP-treated ticks (Fig. 4B). The anti-SBP2 antibody, being polyclonal, did not yield single bands, but produced three bands of varying intensities from 58 to 100 KDa across the samples (Fig. 4C). The three intense bands from the no-treatment controls were fainter in the dsLacZ knockdown except for the 58 KDa one. Similarly, two other bands, except the one at 58 KDa, remained faint in the SBP2 and SELENOP knocked-down tick tissues (Fig. 4C). The GAPDH protein level across the tick tissues was lower in the dsSBP2- and dsSELENOP-injected female ticks than in the no-treatment and dsLacZ-injected females (Fig. 4D).

3.4.3. Impact on oxidative stress—Contrary to our expectations, we did not see an increase in lipid peroxidation for *R. parkeri* ($p = 0.19$). Likewise, no lipid peroxidation was detected after silencing tick *SBP2* ($p = 0.09$) and *SELENOP* ($p = 0.35$) (Fig.S5).

3.4.4. Impact on selenium concentration—Decreased selenium levels were detected in the *SBP2* and *SELENOP* gene-silenced ticks, but the result was only statistically significant for the *SELELNOP* knockdowns ($p = 0.04$) (Fig. 2D). The selenium level decreased in the SBP2- and SELENOP-silenced ticks to 0.073 μg/g and 0.053 mg/g, respectively, from 0.105 μg/g in the dsLacZ ticks (Fig. 2D).

3.4.5. Impact on tick microbiota colonization—The abundance of *R. parkeri* was unchanged in the gut tissues and salivary glands of the *SBP2* and *SELENOP* knockdown ticks (Fig. 5A). The FLE load remained unchanged in the *SBP2* knockdown ticks, but increased significantly in the *SELNEOP*-silenced gut tissues ($p = 0.01$), while the salivary gland load remained unchanged ($p = 0.09$) (Fig. 5B). The CMM load increased significantly in the *SBP2*-silenced gut tissues ($p = 0.009$) and the *SELENOP*-silenced gut tissues ($p = 0.01$), but remained unchanged in the salivary glands (Fig. 5C). However, the overall bacterial load (estimated via 16S rRNA gene analysis) showed significantly increased levels in the *SBP2*- ($p = 0.01$) and *SELENOP*-silenced midguts ($p = 0.02$) (Fig. 5D). Intriguingly, the total bacterial load significantly increased in the *SELENOP*-silenced salivary glands ($p = 0.03$) (Fig. 5D).

3.4.6. Impact on tick fecundity and transovarial R. parkeri transmission—We noticed that tick engorgement remained unaffected ($p = 0.85$), but that the egg conversion ratio was significantly impaired ($p = 0.0004$). Furthermore, the SBP2-silenced ticks laid non-hatch-able eggs (Fig. 6A–D, Table 1). *R. parkeri* transovarial transmission was estimated in the eggs collected post-oviposition (25 days after repletion). The results showed that *R. parkeri* transmission decreased significantly in the *SBP2*-silenced ticks ($p = 0.033$) compared with the control (Fig. 6E). Tick *SELENOP* silencing had no significant effect on the egg conversion ratio (Table 1), and had no significant impact on *R. parkeri* transovarial transmission ($p = 0.78$) (Fig. 6F).

4. Discussion

We functionally characterized tick SBP2 and SELENOP using a reverse genetics approach (RNAi) in *A. maculatum*, a known vector of *R. parkeri*. Functional characterization of *SBP2*, a gene that plays a critical role in selenoprotein synthesis, and *SELENOP*, a gene known to play role in selenium transport, yielded important information about their effects on tick fecundity, pathogen replication, trans-ovarial transmission, and the overall selenium concentration inside ticks.

Computationally, we showed the presence of sequence conservation in tick SBP2 and SELENOP and their respective homologs in vertebrates and invertebrates using multiple sequence alignments and the phylogenetic relationships among them (Figs S1–4). The conserved cysteine residues are represented in boxes with red borders; they are important for the binding capacity of the SECIS region to selenoprotein mRNAs (Bubenik et al., 2015). A

search of the National Center for Biotechnology Information (NCBI) conserved domains database (CDD) hit the binding domain of the ribosomal protein 7Ae/L30e/S12e/Gadd45 family and the RNA binding domain in the overlapped region shaded grey in the alignment (Fig. S1) (Marchler-Bauer et al., 2015). The SBP2 domain structure and ribosomal binding studies showed that the SBP2–ribosome interaction occurs via *28S rRNA*. The studies showed that RNA binding was a prerequisite for Sec insertion, and the importance of the glycine residue in RNA binding ability in the region between amino acids 517–777 in rat SBP2 (Copeland et al., 2001). The evolutionary linkage for SBP2 across the taxa revealed a separate clade for tick species, which differs from the other arthropods and invertebrates. *SBP2* binds to the 3′ untranslated region (UTR) of selenoprotein mRNA, which has a specific stem loop structure like that seen in phospholipid hydroperoxide glutathione peroxidase, and direct cross linking and competition experiments have shown that SBP2 binds to the UTR between nucleotides 82 and 104 (Copeland and Driscoll, 1999).

By not having signal peptide sequences that match the N-ter-minal selenocysteine residue in vertebrate SELENOPs, tick SELENOP is unique (Fig. S2). NCBI CDD databases searches identified the putative domain (SelP_N) in SELENOP from A. maculatum. SELE-NOPb from zebra fish contains only one selenocysteine (U) at its N-terminus (Fig. S2). The number of selenocysteine residues among vertebrate SELENOP molecules varies from 10 to 17. Specifically, rat, mouse, and human SELENOP molecules contain 10 selenocysteine residues (one in the N-terminal and 9 in the C-terminus), whereas zebra fish (SELENOPa) contains 17 selenocysteine residues (one in the N-terminal and 16 in the C-terminus) (Fig. S2).

SBP2, SEPHS2 and *SELENOP* genes were transcriptionally active across the blood meal period (Fig. 1A–C), and were differentially expressed during the pathogen infection (Fig. 1D). The increased *SBP2* and *SEPHS2* transcript levels in the 8-day fed gut tissues and the increased SELENOP transcript levels at 4 dpi showed their importance in blood meal processing, but they probably responded to the increased selenium level in ticks after the blood became imbibed (Fig. 1A–C and Fig. 2B–C). The transcriptional expression of *SBP2* and *SELENOP* increased significantly in the presence of the pathogen infections, suggesting that an increased antioxidant capacity is required in tick salivary glands when *R. parkeri* is present as this pathogen increases the overall oxidative stress (as was measured by lipid peroxidation) (Fig. S5).

The selenium level increased significantly upon blood meal consumption in all the tick species we tested, but it remained unchanged in the infected male *A. maculatum* ticks. Our results support the hypothesis that host blood is the source of selenium in the ticks, and that ticks utilize it for selenoprotein synthesis. Selenium is maintained in its inorganic form (selenite, selenate) through the action of glutathione reductase or thioredoxin reductase. Selenium is also maintained in its organic form (like selenomethionine and selenocysteine) via selenocysteine lysis activity or trans-selenation. These activities are required for selenide production, and selenium, which is a substrate for *SEPHS2*, is required for conversion into selenophosphate, a selenium donor in selenoprotein synthesis (Cardoso et al., 2015; Veres et al., 1994). The 40-fold plus upregulation of SEPHS2 at the fast feeding stage and significantly high selenium levels in blood-fed ticks suggest there is high sele-nophosphate

synthesis. This is supported by high expression of SELENOP, a vital selenoprotein that plays a role in selenium transport to different mammalian body tissues (Richardson, 2005). Selenium plays a maintenance role in the body; therefore, when SELENOP is expressed it indicates that an optimal nutritional intake of selenium is occurring in individuals (Hoeflich et al., 2010). Furthermore, in healthy euthyroid postmenopausal women, bone turnover and bone mineral density have been found to be independently related to selenium status (Hoeg et al., 2012).

To further elucidate the functional roles of SBP2 and SELENOP, their encoded genes were individually silenced in Rp + *A. maculatum*. Only SELENOO was significantly upregulated, and the transcript levels of the other selenoproteins remained unchanged or depleted in the SBP2-silenced ticks, suggesting that downstream selenoprotein synthesis was significantly impacted, as was reported in humans with SBP2 mutations (Schoenmakers et al., 2010). Unlike the SBP2 knockdown, SELENOP silencing resulted in upregulation of *SEPHS2, SELENOO, TXNRD2* and *Cu/Zn-SOD*, which supports the hierarchical impact of selenium transportation to different selenoproteins reported previously (Reszka et al., 2012). In western blots, both SBP2 and SELENOP knockdown ticks showed depletion of SELENOP, and this correlated with the significant depletion of *SELENOP* and *SBP2* and in the *SELENOP*-silenced ticks (Fig. 4B). The SELENOP epitope recognized in ticks further confirmed the presence of this protein in ticks, but we cannot rule out the possibility that the ticks may contain an incomplete sequence. Because of the polyclonal nature of the SBP2 antibody, it is possible that we observed non-specific antibody binding or cross-reactivity as multiple bands of different molecular weights were observed. We witnessed greater band intensity in both dsRNA-SBP2 and dsRNA-SELENOP samples at the ~45kD position as compared with the control samples, possibly via a tick compensatory mechanism activated in response to down-regulating these genes (Fig. 4C).

In both *SBP2*- and *SELENOP*-silenced tick tissues, *SELENOO*, which is an antioxidant and a putative mitochondrial kinase, remained highly expressed and probably compensated for the reduced oxidative balance in the mitochondria (Han et al., 2014). Tick *Cu/Zn-SOD* plays a role in detoxifying the superoxide radicals produced by ticks free of or infected with *R. parkeri* (Crispell et al., 2016). The action of *Cu/Zn-SOD* may also compensate for *SBP2* or *SELENOP* depletion. The thioredoxin-like function of *SELENOP* (based on its Sec-X-X-C domain) means that the upregulated TXNRD2 gene probably compensates for the depletion of SELENOP (Mostert, 2000). The upregulation of *Cu/Zn-SOD*, but depletion of *catalase*, results in super-oxide radicals being generated The upregulation of *Cu/Zn-SOD*, but depletion of *catalase*, results in super-oxide radicals being generated following depletion of *SELENOP* (Burk et al., 2003). This could be the reason for the unchanged lipid peroxidation levels in the samples. The significant depletion of *SELENOS* from *SELENOP*-silencing probably induces stress on the ER, which impacts on its protein folding functions (Shchedrina et al., 2010b). The transcriptional expression levels of *SELENOM, SELE-NOK, SELENON, SELENOT* and *eEFsec* did not change after *SELENOP* silencing, which highlights the lower impact of this protein on the biosynthesis of these selenoproteins. A progressively higher demand for selenoproteins occurred in the tick gut tissues via increased expression levels of *SEPHS2* and *SBP2* after *SELENOP* depletion.

The protective functions of *SBP2* and *SELENOP* against oxidative and lipid peroxidation provides a redox balance in ticks (Burk et al., 2003; Copeland and Driscoll, 1999). Unlike the known anti-apoptotic function of SBP2 (Papp et al., 2010) and anti-peroxidation property of *SELENOP* (Rock and Moos, 2010), silencing of the tick *SBP2* and *SELENOP* did not result in any peroxidation. The reduction of *R. parkeri* levels in *SBP2*- and *SELENOP*-deficient tick salivary glands was likely compensated for by higher level of symbionts such as CMM, FLE, and others, thus resulting in an increased bacterial load in the SBP2- and SELENOP-silenced tick salivary glands (Fig. 5).

Depletion of *SBP2, SELENOP* and selenium possibly caused the reduced egg laying we observed in the ticks (lower fecundity), a result similar to that reported for *Drosophila* feeding on a selenium-deficient diet (Martin-Romero et al., 2001). Embryonic lethality was shown by the *SBP2*-deficient tick laid eggs failing to hatch. Reduced selenoprotein expression in *SBP2* knockout mice and humans with *SBP2* mutations also result in embryonic lethality (Schoenmakers et al., 2010; Seeher et al., 2014). The depleted selenium levels in the *SBP2*- or *SELENOP* silenced ticks are consistent with the results from studies on knockout mice. The *SELENOP* knockout mice showed reduced transportation of selenium from hepatocytes to the peripheral tissues (Schoenmakers et al., 2010; Seeher et al., 2014). Conversely, the selenocysteines found in SELENOP were replaceable by cysteines (in some among 10 mice) if a selenium-deficient diet was provided (Hill et al., 2012; Turanov et al., 2015). In knockout mice, depleted levels or reduced selenium utilization was also reported for Gpx1, another selenoprotein (Kasaikina et al., 2011b). An overall decrease in tick engorgement weight in the *SELENOP*-deficient ticks supports the potential role of SELENOP in tick hematophagy and oviposition. Reduced transovarial (vertical) transmission of *R. parkeri* by silencing *SBP2* and *SELENOP* and an accompanying reduction in egg laying may provide a novel control strategy for tick-borne pathogens and their vectors. We propose that arthropods like ticks may be controlled using a conserved motif in SBP2 or SELENOP as a vaccine target.

In conclusion, our data provide important insights into the role of SBP2 in tick selenoproteome synthesis, tick fecundity, reproductive fitness (egg development), and transovarial transmission of *R. parkeri*. Likewise, tick SELENOP, along with tick SBP2, maintains tick selenium levels via their roles in selenoprotein synthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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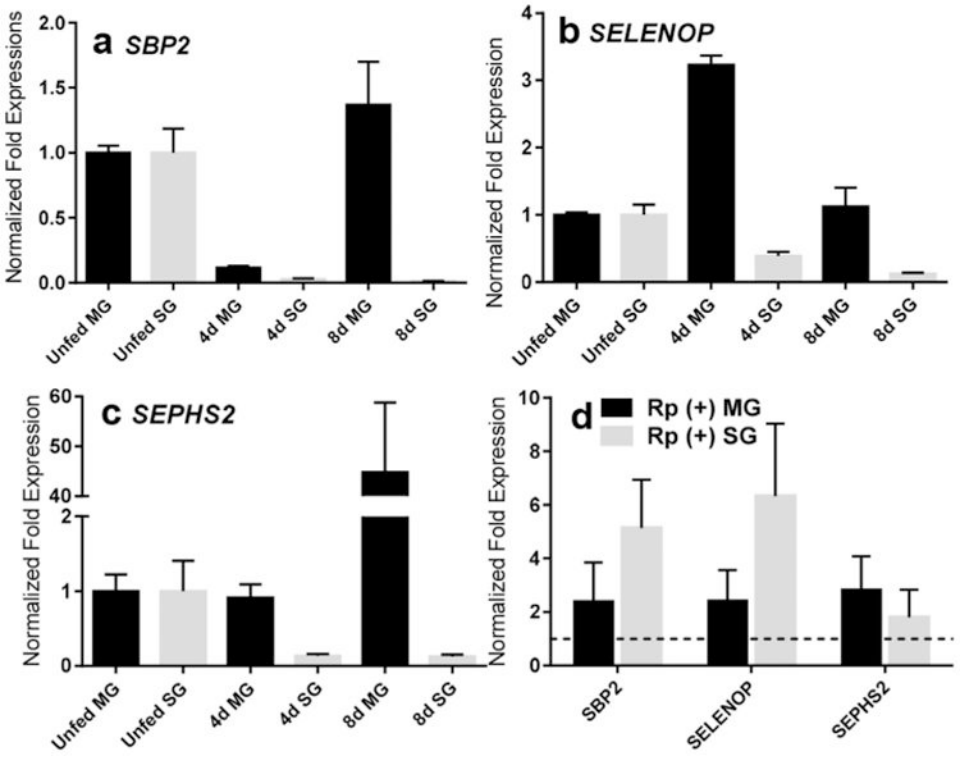

Fig. 1. Transcriptional expression of SECIS binding protein 2 (*SBP2*), selenoprotein P (*SELENOP*) and selenophosphate synthetase (*SEPHS2*) genes post-blood meal during *Rickettsia parkeri* infection in *Amblyomma maculatum*

Transcriptional gene expression for (A) *SBP2*, (B) *SELENOP*, and (C) *SEPHS2* was estimated in unfed ticks, slow feeding (4-day) ticks, and fast feeding (8-day) ticks in adult female *A. maculatum*. The expression value for the unfed ticks was set to 1 for reference. (D) Tick *SBP2, SELENOP* and *SEPHS2* genes were up-regulated in *R. parkeri*-infected tick midgut and salivary glands tissues. The transcriptional activities were normalized against tick glyceraldehyde 3-phosphate dehydrogenase.

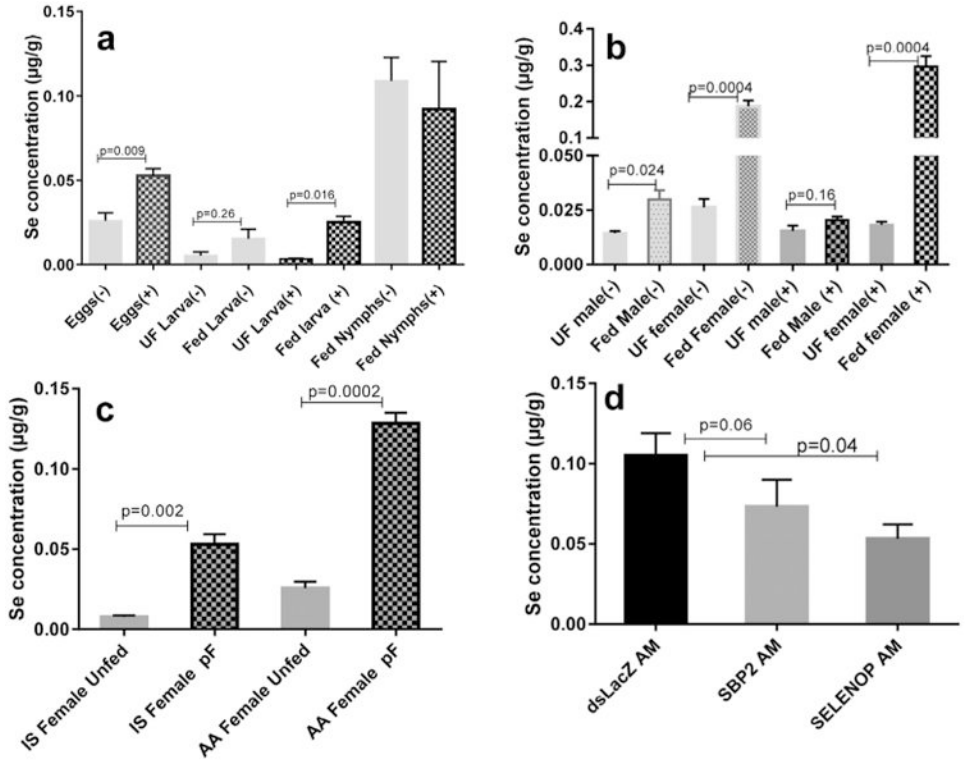

Fig. 2. Selenium levels in immature and mature ticks

Selenium levels across (A) embryonic and immature stages of blood fed and unfed (UF) ticks, (B) adult fed and UF male and female *Amblyomma maculatum* ticks with (+) or without (–) *Rickettsia parkeri* infection before and after a blood meal. (C) Selenium levels in UF and 8-day partially fed (pF) female *Ixodes scapularis* (IS) and *Amblyomma americanum* (AA) ticks. (D) Selenium concentrations in the control, and in the *SBP2*- and *SELENOP*-*silenced Amblyomma maculatum* (AM) ticks. At least three biological replicates were used to estimate the selenium levels via the inductively coupled plasma mass spectrometry (ICP-MS) technique.

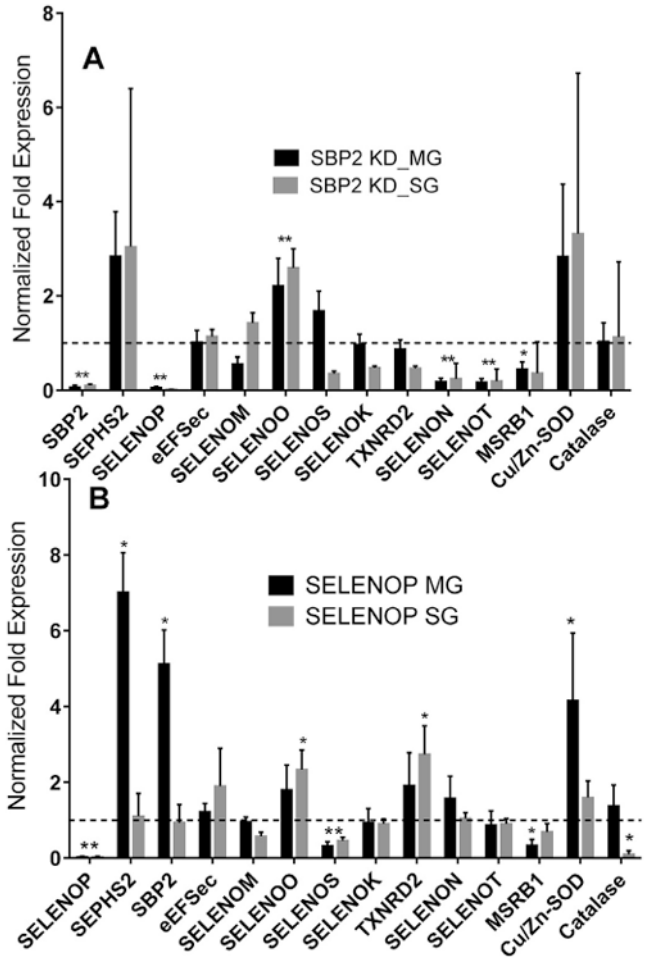

Fig. 3. SECIS binding protein 2 (*SBP2*) gene (A) and selenoprotein P (*SELENOP*) gene (B) knockdowns in *Amblyomma maculatum*

Transcriptional expression of the selenogenes was assessed in *SBP2* and *SELENOP* knockdown tick tissues. Quantitative reverse transcriptase PCR was used to determine the transcriptional expression levels of the tick selenoproteins using tick *GAPDH* as the reference gene. The expression levels of the target genes in the control samples were set to 1.

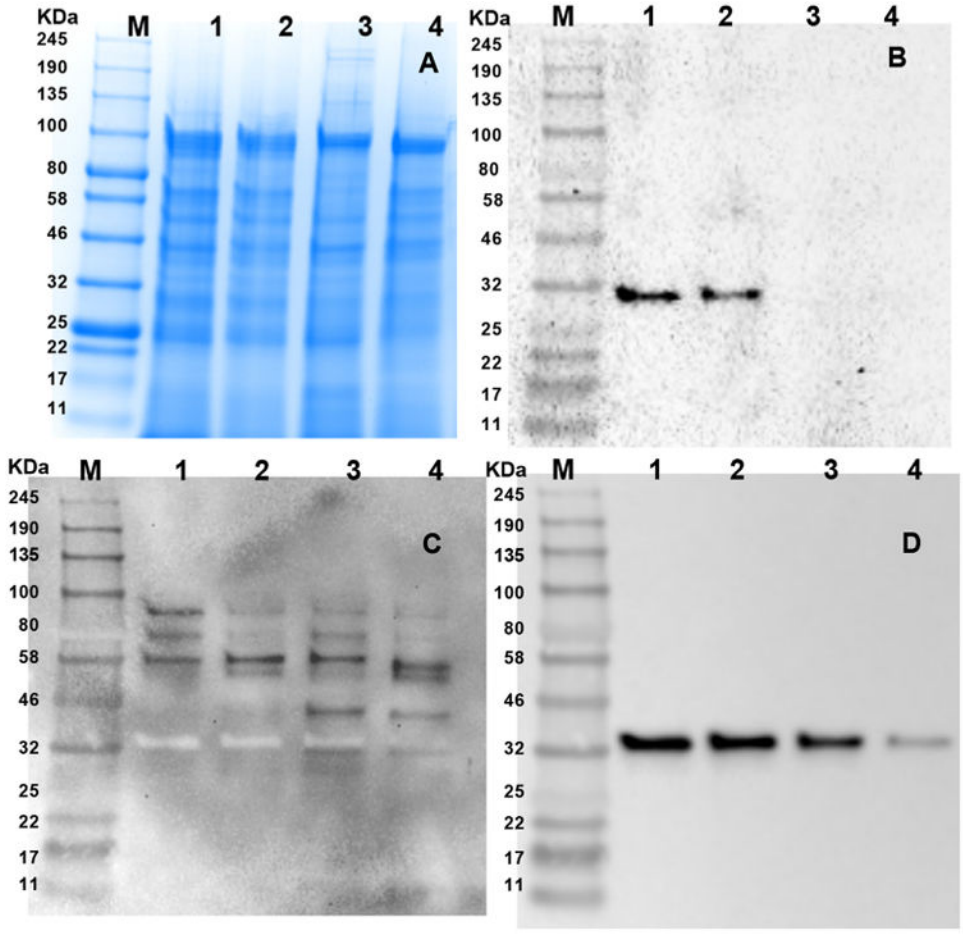

Fig. 4. Immune-blot analysis of SECIS binding protein 2 (SBP2) and selenoprotein P (SELENOP) proteins in *Amblyomma maculatum*

(A) SDS-PAGE gel (4–20%) stained with GelCode Blue; (B) the corresponding immunoblots showing cross reactivity to an anti-human SELENOP antibody; (C) immunoblot incubated with SBP2 antibodies and (D) immunoblot incubated with anti-glyceraldehyde phosphate dehydrogenase monoclonal antibodies. M: Broad range molecular weight protein standard. Lanes 1 and 2 show the no-treatment ticks and the dsLacZ-injected *A. maculatum* female ticks, respectively. Lanes 3 and 4 show dsSBP2-and dsSELENOP-injected *A. maculatum* female ticks, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

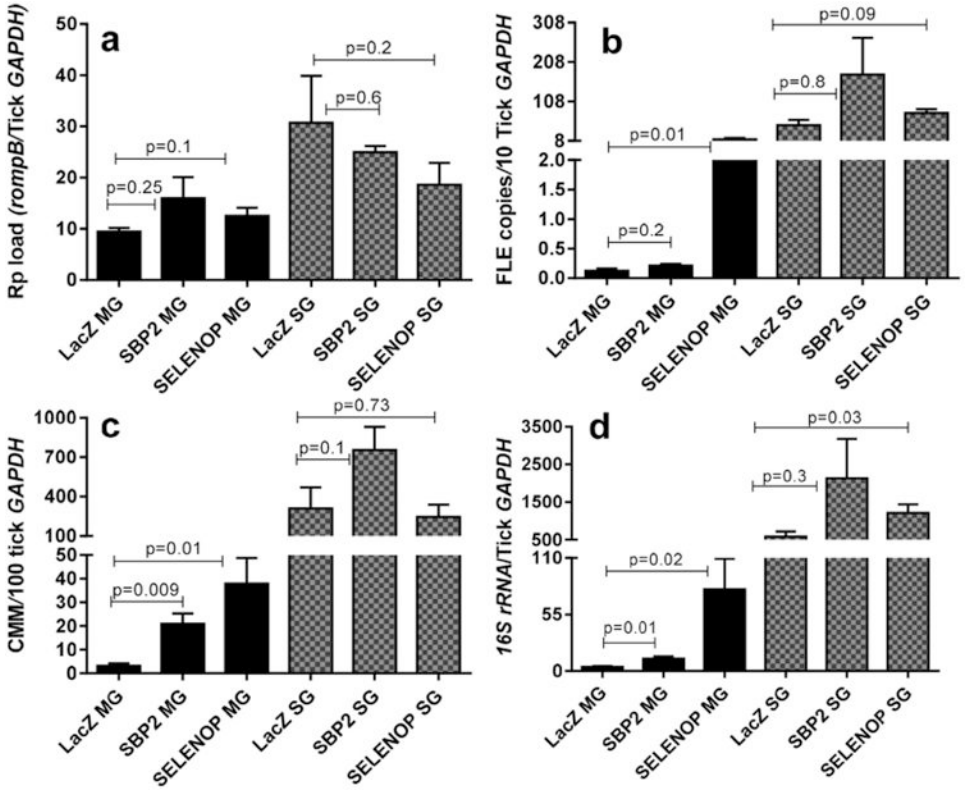

Fig. 5. *Rickettsia parkeri* and symbiont loads in tick SECIS binding protein 2 (*SBP2*) gene and selenoprotein P (*SELENOP*) gene knockdown tissues

(A) *R. parkeri* load in tick midguts (MG) and salivary glands (SG). (B) Francisella-like endosymbiont (FLE) load in dsRNA-injected tick tissues 5 days after the injections. (C) *Candidatus* Midichloria mitochondrii (CMM) load in dsRNA-injected tick tissues. (D) Bacterial load in midguts and salivary glands in the dsRNA-injected ticks, as determined by *16S rRNA* analysis.

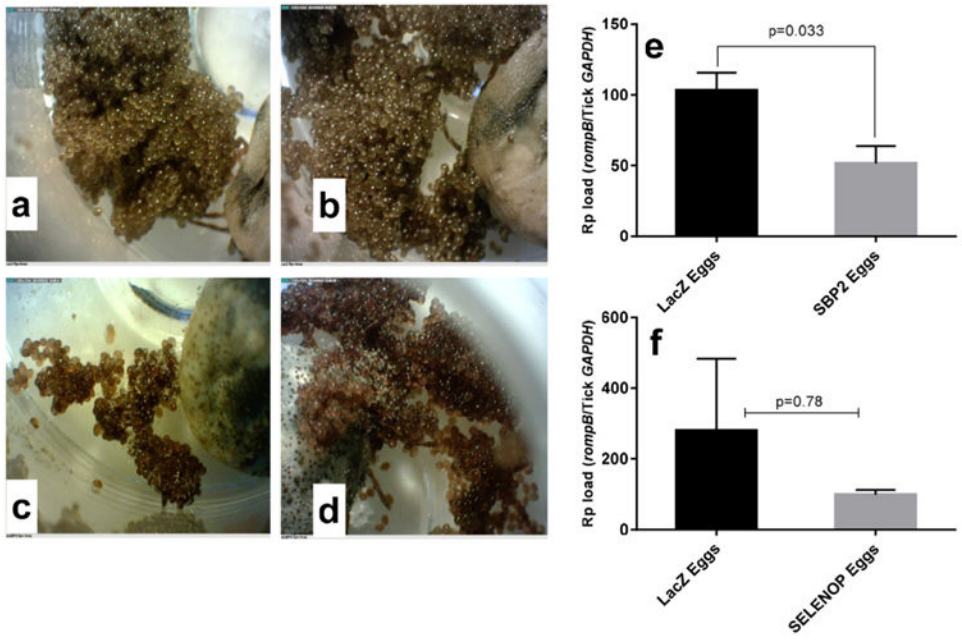

Fig. 6. Impact of the SECIS binding protein 2 (SBP2) on tick fecundity and transovarial transmission of *Rickettsia parkeri* from *SBP2* and *SELENOP* gene knockdowns

Eggs from the control (dsLacZ-injected) (a, b) and dsSBP2 injected (c, d) ticks. (e, f) *R. parkeri* load in tick eggs after *SBP2* and *SELENOP* silencing.

Table 1

Phenotypes of *dsLacZ* (irrelevant control), double-stranded SECIS binding protein (ds*SBP2*), and *SELENOP* (ds*SELENOP*)-injected *Amblyomma maculatum* ticks (mean ± SEM)

Weights are in mg. Egg conversion was calculated as the ratio of total tick egg weights at egg laying completion to gravid female tick weights when egg laying started.

Group	Infection status	No. of ticks	Engorged (Replete) tick weight (Range)	Egg mass (Range)	Egg conversion ratio (Range)
dsLacZ	Rp + Ticks	25	769 ± 46 (435–1090)	325 ± 106 (38–497)	0.55 ± 0.04 (0.44–0.67)
dsSBP2	Rp + Ticks	25	754 ± 38 (632–904)	83 ± 56 (22–253)	0.11 ± 0.08 (0.034–0.345)
dsSELENOP	Rp + Ticks	15	539 (504–574)	0–312	0.54