Knockdown of the *Rhipicephalus microplus* Cytochrome c Oxidase Subunit III Gene Is Associated with a Failure of *Anaplasma marginale* Transmission

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Abstract

*Rhipicephalus microplus* is an obligate hematophagous ectoparasite of cattle and an important biological vector of *Anaplasma marginale* in tropical and subtropical regions. The primary determinants for *A. marginale* transmission are infection of the tick gut, followed by infection of salivary glands. Transmission of *A. marginale* to cattle occurs via infected saliva delivered during tick feeding. Interference in colonization of either the tick gut or salivary glands can affect transmission of *A. marginale* to naïve animals. In this study, we used the tick embryonic cell line BME26 to identify genes that are modulated in response to *A. marginale* infection. Suppression-subtractive hybridization libraries (SSH) were constructed, and five up-regulated genes (glutathione S-transferase (GST), cytochrome c oxidase sub III (COXIII), dynein (DYN), synaptobrevin (SYN) and phosphatidylinositol-3,4,5-triphosphate 3-phosphatase (PHOS)) were selected as targets for functional in *vivo* genomic analysis. RNA interference (RNAi) was used to determine the effect of tick gene knockdown on *A. marginale* acquisition and transmission. Although RNAi consistently knocked down all individually examined tick genes in infected tick guts and salivary glands, only the group of ticks injected with dsCOXIII failed to transmit *A. marginale* to naïve calves. To our knowledge, this is the first report demonstrating that RNAi of a tick gene is associated with a failure of *A. marginale* transmission.

Introduction

Ticks and tick-borne pathogens, including *Anaplasma marginale*, cause significant economic losses for the livestock industry worldwide. These economic losses are associated with the following: 1) reduction of milk and meat production; 2) temporary infertility; 3) treatment costs; 4) mortality; and 5) secondary bacterial infection in the open wounds caused by tick feeding [1]. *Anaplasma marginale* is an obligate gram-negative bacterium transmitted by ticks, including *Rhipicephalus* species. In Latin America, it is estimated that bovine anaplasmosis and babesiosis cause annual economic losses exceeding US$ 800 million [2]. In endemic regions, anaplasmosis control strategies include the use of a live-attenuated vaccine, a killed vaccine, antibiotic prophylaxis and/or tick control measures [3,4]. Vaccines are the most effective method for controlling disease and induce protective immunity that prevents acute bacteremia. However, vaccines do not prevent *A. marginale* infection, and infected animals can serve as reservoirs for tick transmission [1,4].

Ticks are an efficient biological vector of *A. marginale* and acquire the bacteria from acutely or persistently infected animals [5]. There is no transovarial transmission of *A. marginale* from female ticks to tick offspring [6,7], and transstadial and intrastadial transmission by male ticks are considered the most important means of *A. marginale* transmission [8,9]. In the tick, *A. marginale* first infects gut epithelial cells. After colonization of the tick gut, the bacteria migrate through the hemocoel to infect tick salivary glands [10]. Transmission occurs via saliva when infected ticks feed on an uninfected host [11].

Cellular and molecular interactions between *A. marginale* and ticks are poorly understood. Tick cell lines, including ISE6, IDE8 (derived from *Ixodes scapularis*) and BME26 (derived from *R. microplus*), have been used in transcriptional and protein expression studies to examine differential tick responses to *A. marginale* infection [12–16]. Those studies demonstrated that *A. marginale* infection alters normal tick gene transcription and protein expression. In the current study, we identified differentially regulated tick genes in response to *A. marginale* infection in a BME26 cell line by suppression-subtractive hybridization. A subset of differentially regulated tick genes was selected based on functional annotation and targeted for *in vivo* gene knockdown.
studies using RNAi. We examined the impact of *R. microplus* gene knockdown on *A. marginale* acquisition and transmission.

**Materials and Methods**

**Ethics Statement**

All experiments involving animals were approved by the University of Idaho, Institutional Animal Care and Use and Biosafety Committees (Protocol Numbers, IACUC: 2013-66, Biosafety: B-010-13) in accordance with institutional guidelines based on the U.S. National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

**Cattle infection by Anaplasma marginale** and male tick rearing

Eleven spleen-intact, age-matched (3-month old) Holstein calves were used in this study; two for rearing ticks (C38088 and C40440), two for acquisition feeding experiments (C37837 and C39306) and seven for transmission feeding experiments (C38098, C38099, C38100, C38101, C38118, C40444 and C40456). These calves were confirmed to be free of *A. marginale* by MSP5-CI-ELISA [17] and *msp5*-nested PCR [18]. The calves used in the acquisition feeding experiments were inoculated with ~10^7 *A. marginale*-infected erythrocytes (St. Maries strain) as described previously [11].

To rear male ticks, approximately 40,000 larvae from 2.0 grams of *R. microplus* eggs were placed under a cloth patch on a naive calf. On day 14, engorged nymphs were manually removed from the calf with forceps and held in an incubator at 26°C and 92% relative humidity until molting into adults.

**Infection of BME26 with Anaplasma marginale**

BME26, an embryonic cell line derived from the cattle fever tick, *Rhipicephalus microplus*, was cultured in L-15B300 medium as previously described [19]. Approximately 3.5 x 10^7 cells were subcultured into eight new flasks and incubated for 24 h, after which time 5 ml of the culture medium in each flask was replaced with cultured into eight new flasks and incubated for 24 h, after which time 5 ml of the culture medium in each flask was replaced with transfected medium (C38098, C38099, C38100, C38101, C38118, C40444 and C40456). These calves were confirmed to be free of *A. marginale* by MSP5-CI-ELISA [17] and *msp5*-nested PCR [18]. The calves used in the acquisition feeding experiments were inoculated with ~10^7 *A. marginale*-infected erythrocytes (St. Maries strain) as described previously [11].

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**Identification of differentially regulated tick genes during A. marginale infection**

Suppression-subtractive hybridization (SSH) was performed using the Clontech PCR-Select cDNA Subtraction Kit [21], and cDNA was prepared using the SMARTer Pico PCR cDNA Synthesis Kit, according to the manufacturer’s instructions (Clontech, Palo Alto, CA, USA). To identify tick genes that are up-or down-regulated as a consequence of *A. marginale* infection, forward and reverse SSH libraries were constructed as follows: pools of 2 μg total RNA were prepared from uninfected and *A. marginale*-infected BME26 cells. After RNA digestion, cDNA from both groups were ligated to adaptors. The forward SSH library was made by hybridizing adapter ligated cDNA from BME26 cells infected with *A. marginale* as the tester in the presence of an excess of cDNA from uninfected cells as the driver. The reverse SSH library was made in the same manner, but in this case, the adapter ligated cDNA from uninfected BME26 cells was used as the tester, and infected cells cDNA as the driver. The forward and reverse libraries were used to identify up- or down-regulated BME26 transcripts, respectively, in response to *A. marginale* infection. Differentially expressed cDNAs were PCR amplified with Advantage PCR Polymerase Mix (Clontech), cloned using the pGEM-T Easy Vector System (Promega, Madison, WI, USA), and transformed into XL1-Blue E. coli cells plated on LB with ampicillin, X-gal and IPTG. Individual colonies were randomly selected from each library and inoculated into LB medium supplemented with ampicillin and incubated overnight. Plasmids were purified using the Wizard SV 96 Plasmid DNA Purification System (Promega), and plasmid inserts were PCR amplified and sequenced on an ABI PRISM 3700 DNA sequencer (Applied Biosystems, Foster City, CA, USA) using the BigDye Terminator cycle sequencing protocol (Applied Biosystems).

**Functional annotation of assembled sequences**

High quality sequences were obtained from both the forward and reverse SSH libraries and assembled together in one run into unique contigs by the CAP3 assembler (Contig Assembly Program, Version 3) as previously described [22]. The library origin of the reads assembled in each up- or down-regulated contig was tracked, and except in the case of glutathione S-transferase, the contigs assembled from reads from only one library. Both manual and automatic annotations were performed to search for similarity among the sequences obtained in both SSH libraries and other libraries deposited in public databases (GenBank and Swiss-Prot), as well as all to sequences available from *R. microplus*. For these purpose two data banks of DNA sequences were made: one created from public data of *R. microplus* (GenBank) ESTs (RHIPI2011 database), and another constructed after the analysis of an extensive transcriptome (seven organs/tissues of *R. microplus*, harvested in various stages of development) (Solexa-ASB database). In this last case, the cDNA generated was sequenced by Illumina technology, and the complete analysis of the results obtained will be the subject of another publication. To search for conserved functions, the sequences were analyzed by RPS Blast against several conserved domain databases, including GO (Gene Ontology) [23], SignalP (Signal Peptide) [24], KOG (Eukaryotic Orthologous Groups) [25], CDD (Conserved Domain Databases) [26], PFAM (Protein family database) [27], SMART (Simple Modular Architecture Research Tool) [28] and MIT-PLA (Mitochondrial and Plasmid Sequences database), available from NCBI. The final assembly output was piped into a tab-delimited file that was imported into an Excel spreadsheet, which includes functional classification of each assembled up and down contigs, as described previously [29].

**Quantification of tick gene expression and A. marginale in R. microplus guts and salivary glands**

Total RNA and genomic DNA was extracted from pools of 5 adult male tick guts and salivary glands using AllPrep DNA/RNA Micro (QIAGEN, Valencia, CA, USA) according to the manufacturer’s instructions. Total RNA samples were treated with DNase I (Invitrogen), and cDNA was synthesized with AllPrep DNA/RNA Micro (Bio-Rad, Hercules, CA, USA).

To quantify the tick gene expression, real time quantitative PCR (qPCR) was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad) and Express SYBR Green ER Supermix (Invitrogen), using cDNA as template and specific primers (Table S1). The qPCR cycling conditions consisted of an initial denaturation of 95°C for 30 sec followed by 40 cycles at 95°C denaturation for 5 sec, and annealing/extension at 60°C for
analysed for each sample. The CFX Manager Software (Bio-Rad)
program: 10 minutes at 95
thermocycler
Briefly, the reactions were performed on a CFX96 Real-Time
primers (Table S1) and a TaqMan probe targeting
were quantified by qPCR using gDNA as template, specific
buffered EDTA) samples [24].

The total number of Anaplasma in tick guts and salivary glands
were quantified by qPCR using gDNA as template, specific
primers (Table S1) and a TaqMan probe targeting
Ct infected - mean Ct control (uninfected) samples or

Preparation of double-stranded RNA
Glutathione S-transferase (GST), cytochrome c oxidase subunit
III (COXIII), dynein (DYN), synaptobrevin (SYN) and phosphatidyl
inositol-3,4,5-triphosphate 3-phosphatase (PHOS) were se-
lected for knockdown studies using RNA interference technique.
Primer sets for each gene were designed (Table S1) using Primer3
software [32,33]. Tick guts and salivary glands from adult males
were used as a template for generating target gene amplicons.
Amplicons were cloned into the TOPO TA Cloning Kit Dual Promoter
(pGRII-TOPO) (Invitrogen). The MEGAscrip Transcription Kit was used for dsRNA synthesis following the
manufacturer’s protocol (Ambion, Austin, TX, USA). The dsRNA
molecules were purified, quantified by spectrophotometry, ana-
lyzed by gel electrophoresis, and stored at −20°C.

Transmission trials of A. marginale
Five cohorts of 200 freshly molting male ticks for each
experimental group were injected with dsGST, dsCOXIII,
dsDYN+SYN, dsPHOS and buffered 0.1 mM EDTA in trial 1. Ticks were placed ventrally up side on double-sided tape for
injections. They were injected with 2×10^{11} molecules of dsRNA suspended in buffered 0.1 mM EDTA through the coxal
membrane at the base of the 4th leg on the right ventral side
using a Hamilton syringe with a 36 gauge needle and a
microprocessor-controlled UMP3 injection pump apparatus
(World Precision Instruments, San Antonio, TX, USA) [34,35]. The control group was injected with 0.1 mM buffered EDTA in a
similar manner. After injection, male ticks were immediately
placed in individual group patches on an infected calf (C37837)
injected ticks and serology [17]. All analyses were made follow-
ing the same methodology described for trial 1.

Statistical analysis
A. marginale infection and tick gene expression data were analyzed by Student’s t-test using SAS 9.2 (SAS Institute, Cary,
NC, USA). If an initial test for equality of variances was not
significant, then a t-test was performed. However, if this initial test
was significant (P≤0.05), then a Satterthwaite t-test was performed
[28]. Tick survival data were analyzed by chi-square tests unless
any contingency table cell contained fewer than 5, in which case
Fisher’s exact test was used instead. The results are expressed as the mean ± S.D. P value≤0.05 was considered significant.

Results
Identification of differentially expressed tick genes
during A. marginale infection
Forward and reverse SSH libraries were constructed to identify
up- or down-regulated tick genes in response to A. marginale
infection using a BME26 cell culture, and 1,536 randomly selected
clones were sequenced. After eliminating clones with poor quality
sequences, 719 expressed sequence tags (EST) were obtained and
used for bioinformatics analysis (Table 1). Clustering and assembly of
ESTs from up-regulated genes resulted in 25 contigs and 106
singletons. Down-regulated genes yielded 211 unique sequences
with 13 contigs sequences and 85 singletons. Automated and manual
annotation was used to search databases (GenBank, Swiss-Prot, RHIPI2011 and Solexa-ASB) for similarity and
putative functions (GO, SignaP, KOG, CDD, PfAM, SMART
and MIT-PLA), and a hyperlinked excel spreadsheet was prepared
with the various information obtained for each contig (Table S2).
The sequences are identified as Rm-contig_number in the first
column, which is hyperlink to its FASTA nucleotide sequence.
Gene ontology assignments were used to obtain more information
about up-regulated genes from the forward SSH library, and
sequences were broadly split into categories of ‘cellular compo-
nent’, ‘molecular function’ or ‘biological process’. A total of 33
sequences were categorized as cellular components (Figure 1A), 35
sequences were assigned to molecular functions (Figure 1B) and 36
sequences were assigned to biological processes (Figure 1C). In
the cellular component category, the majority of transcripts (69%) were classified as intracellular, and 13% were classified as
extracellular. For biological process, the majority of identified
transcripts were related to a cellular metabolic process (50%). In
the molecular function category, most transcripts were related to
binding (40%), catalytic activity (34%), transferase activity (11%),
oxidoreductase activity (6%), ligase activity (3%) or hydrolase
activity (14%).

Selection of tick genes for the genomic functional study
We elected to study five genes with increased transcription
during A. marginale infection in BME26 cells in an in vivo model.
Figure 1. The Gene Ontology analysis. Sequences of up-regulated genes identified by SSH were categorized by (A) cellular component, (B) molecular function and (C) biological process.

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The selection of these tick genes was based on biological functions that could be related to *Anaplasma* colonization in tick cells. These genes include one isoform of glutathione S-transferase (GST) (GenBank accession number KF784792), cytochrome *c* oxidase sub III (COXIII) (GenBank accession number KF784795), dynein (DYN) (GenBank accession number KF784791), synaptobrevin (SYN) (GenBank accession number KF784794) and phosphatidylinositol-3,4,5-triphosphate 3-phosphatase (PHOS) (GenBank accession number KF784793). GST is important in the detoxification of both endogenous and xenobiotic compounds and protection against oxidative stress [36–38]; COXIII is related to mitochondrial metabolism, including ATP production and reactive oxygen species (ROS); DYN is a motor protein involved in the conversion of chemical energy present in molecules of ATP into mechanical energy along microtubules and is involved in intracellular trafficking pathways [39]; SYN is a v-SNARE protein that participates in the exocytosis of proteins [40–42]; and phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase plays an important role in the reorganization of the cytoskeleton, NADPH production and ROS production [43].

In order to confirm the up-regulation of the selected genes from the forward SSH library, the relative gene expression in uninfected and *A. marginale*-infected ticks (harboring 10$^{3.35}$±$^{0.52}$ bacteria per gut and 10$^{1.93}$±$^{0.46}$ bacteria per salivary gland pair) was determined (Figure 2). We observed that GST, COXIII, DYN, SYN and PHOS genes were significantly up-regulated in tick gut and salivary glands during *A. marginale* infection, with the exception of DYN and COXIII in the gut.

### Effect of tick gene knockdown during transmission of *A. marginale*

**Trial 1.** In order to evaluate the outcome of tick gene knockdown on *Anaplasma* acquisition and transmission to calves, RNAi was performed. Groups of freshly molted male ticks were injected with dsGST, dsCOXIII, dsDYN+SYN, dsPHOS and buffered 0.1 mM EDTA. DYN and SYN were injected together because both are related to vesicular trafficking. After injection, male ticks were immediately placed in individual group patches on an infected calf (C37837) and allowed to acquisition feed for 8 days during peak bacteremia. Calf peak bacteremia ranged from 4.2% to 13.6%, and the packed cell volume (PCV) varied from 13% to 38%. The tick survival rate evaluated for the five tick groups at the end of acquisition feeding varied from 30% to 60.5% (Table 2). There was a significant difference in tick survival rates between the dsRNA-injected groups and the control group, in which fewer ticks survived (30%).

### Table 1. Summary of up- and down-regulated genes from *A. marginale*-infected BME26 cells.

| Library          | Up-regulated | Down-regulated |
|------------------|--------------|----------------|
| Total sequences  | 508          | 211            |
| Non-redundant sequences: |       |                |
| Number of singletons | 106  | 85            |
| Number of contigs | 25           | 13             |
| Number of contigs containing: |       |                |
| 2–4 sequences    | 12           | 8              |
| 5–15 sequences ESTs | 8        | 4              |
| >15 sequences    | 5            | 1              |

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Figure 2. Relative gene expression in gut and salivary glands of ticks infected with *A. marginale*. The expression of the cytochrome c oxidase sub III (COXIII), glutathione S-transferase (GST), synaptobrevin (SYN), dynein (DYN) and phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PHOS) genes in guts and salivary glands from *R. microplus* males fed for 8 days on either one uninfected calf (C38080) or one *A. marginale*-infected calf (C37837) was assessed by RT-qPCR. Threshold values were normalized according to the Ct of the reference gene (tubulin). The relative expression level of each gene in infected ticks in relation to uninfected ticks (control) was calculated using the Delta Delta Ct method. The data represent the mean ± S.D. of four pools of 5 guts and salivary glands. An asterisk (*) represent data with differences statistically significant with respect to control (P<0.05).

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Recipient calves were positive for weekly for up to 12 weeks post-tick transmission feeding. All injected with dsDYN loads during transmission feeding, except in salivary glands from dsRNA-injected and control ticks after transmission feeding. In the control, dsSYN, the relative gene expression was 0.29 salivary glands from ticks injected with dsCOXIII, dsDYN and PHOS transcript levels was assessed in the guts and control. The values of the peak bacteria and the package cell volume (PCV) 21% at the 7th week; for calf C38101 (dsGST), the peak bacteremia was 8.0%, PCV 26% at the 7th week; and for calf C38098 (dsPHOS), the peak bacteremia was 3.6%, PCV 20% at the 7th week.

**Trial 2.** Due to failure of *A. marginale* transmission to calf by ticks with COXIII knockdown in the trial 1, a new batch of experiments was set up with dsCOXIII-injected and control ticks. In the second trial, ticks engaged in acquisition feeding on a calf (C39096) with a bacteremia ranging from 0.03% to 6.8% and a PCV of 30% to 38%. There was no significant difference in tick survival rates between the dsCOXIII-injected ticks (41%) and control ticks (39%) after acquisition feeding (Table 2). Similarly, at the end of transmission feeding, no difference was observed between the tick survival rate in dsCOXIII-injected ticks (38%) and control ticks (79%) (Table 2). The COXIII gene knockdown was efficient because the relative gene expression was significantly lower than the control both in the gut (0.31±0.045) and salivary glands (0.63±0.082) (Figure 3A). However, there was no difference between dsCOXIII-injected and control ticks with respect to the *Anaplasma* level in the gut and salivary gland (Figure 3B).

Similarly to the previous trial, the recipient calf (C40456) that received ticks injected with dsCOXIII remained negative by Giemsa-stained blood smears, nPCR and MSP5-ELISA at all-time points tested (Figure 4A), while *A. marginale* was detected by a Giemsa-stained blood smear from the blood of the calf (C40444) within the 4th week post-feeding of control ticks and confirmed by nPCR at the 3rd week. The detection of MSP5 nPCR product from the blood samples at the end of the 12th week is shown in the Figure 4B. The calf seroconverted at the 8th week post-tick transmission and had a peak bacteremia of 8.0% and PCV 26% at the 7th week (Figure 4A).

**Discussion**

In this study, we initially identified a set of *R. microplus* genes that are modulated in response to *A. marginale* infection in two suppression-subtractive hybridization libraries using a BME26 cell line as model. Subsequently, we tested if knocking down selected up-regulated tick genes could affect *A. marginale* infection at the level of the tick gut and salivary glands. In addition, the *A. marginale* infection was evaluated in recipient calves that received gene knocked-down ticks.

We annotated by function 37 tick genes that were up-regulated in response to *A. marginale* infection on BME26 cells (Table S2). Some of these genes have been identified in other “omics” studies [12,16,44]. A proteomic and a transcriptomic approach using IDE8 cells identified the over-expression of several genes, including glutathione S-transferase (GST), cytochrome c oxidase and an unknown protein. Further studies are needed to determine whether this protein is a new target for tick control.

### Table 2. Survival rate of dsRNA injected ticks.

| Target gene | Tick acquisition feeding | Tick acquisition feeding |
|-------------|--------------------------|--------------------------|
| GST         | *51% (102*/200*)         | 94% (77*/82*)            |
| COXIII      | *59% (118*/200*)         | *56% (55*/98*)           |
| DYN + SYN   | *60.5% (121*/200*)       | *74% (75*/101*)          |
| PHOS        | *46.5% (97*/200*)        | 82% (60*/73*)            |
| Control     | 30% (60*/200*)           | 93% (37*/40*)            |
| COXIII      | 41% (61*/150*)           | 88% (36*/41*)            |
| Control     | 39% (59*/150*)           | 79% (31*/39*)            |

Survival rate was calculated by the number of attached ticks *x* 100/placed ticks *b* in calves. Asterisk (*) indicates significant difference (*P*=0.05) between dsRNA injected and control ticks. 

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subunit II and selenoprotein due to *A. marginale* infection, which is similar to our findings [12]. Furthermore, a temporal study of gene expression in adult male *R. microplus* in response to *A. marginale* infection identified several up-regulated genes, including those similar to our library, such as GST and aldehyde dehydrogenase [44]. Finally, a transcriptomic study comparing uninfected and *A. marginale*-infected *R. microplus* salivary glands found induction of the cytochrome *c* oxidase subunit III (COXIII) and a proline-rich protein [16], as identified in our library. However, other genes identified in this previous study, such as the vacuolar H+-ATPase V1 sector subunit, metallothionein, glycine-rich proteins and the von Willebrand factor, have not been detected in the current study. Differences between our results and Zivkovic et al [16] results could be due to 1) the use of an embryonic cell line as

Figure 3. Gene knockdown efficiency and *A. marginale* levels on dsRNA injected ticks after transmission feeding. (A) The expression of the cytochrome *c* oxidase sub III (COXIII), glutathione S-transferase (GST), synaptobrevin (SYN), dynein (DYN) and phosphatidylinositol-3,4,5-triphosphate 3-phosphatase (PHOS) genes in guts and salivary glands from dsRNA injected and control ticks (injection of an equal volume of 0.1 mM EDTA) were evaluated by RT-qPCR. The relative expression level of each gene in dsRNA-injected ticks in relation to EDTA-injected ticks (control) was calculated by the Delta Delta Ct method. Threshold values were normalized according to the Ct of the reference gene (tubulin). The data represent the mean ± S.D. of four pools of 5 guts and salivary glands. An asterisk (*) represent data with differences statistically significant with respect to control (*P*<0.05). (B) The bacteria number was determined by qPCR using specific primers and a TaqMan probe for *msp5* gene.

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opposed to infected tick organs, 2) divergence of *A. marginale* strains, 3) differences in experimental infection conditions used between the studies

After an accurate analysis of up-regulated BME26 genes, five genes were selected for a functional genomic study based on their biological role and putative connection with *Anaplasma* colonization: GST is involved with detoxification processes and promotes cell protection against oxidative stress; COXIII is associated with mitochondrial metabolism that results in ATP or reactive oxygen species (ROS) generation; DYN and SYN are related to intracellular trafficking pathways and may be associated with bacteria survival and replication within tick cell vacuoles; and PHOS is involved in several cellular functions, such as cytoskeleton reorganization and NADPH and ROS production. Among these genes, only GST and COXIII have been previously shown to be up-regulated in cell culture and in ticks in response to *A. marginale* infection and during blood feeding [12,15,16,44–46].

The over-expression of GST in IDE8 tick cells and D. variabilis salivary glands by *A. marginale* infection was suggested as a response to reduce the oxidative stress caused by pathogen infection and thus increases pathogen multiplication in tick cells [12]. In ISE6 tick cells, GST was also up-regulated [15]. After nine days of *A. marginale* infection, a higher amount of GST was expressed in the *R. microplus* gut than in the salivary gland [44]. A similar modulation of the expression profile was observed for the COXIII gene in IDE8 cells in response to *A. marginale* infection [12], and the up regulation was also verified in the salivary glands of adult male *R. microplus* [16,44]. Up-regulation of cytochrome *c* oxidase during pathogen infection could be associated with an increase in the metabolic demands placed on arthropod vectors by the pathogens. A previous study demonstrated that cytochrome *c* oxidase subunit VIb was up-regulated in *Anopheles gambiae* upon *Plasmodium berghei* infection [45]. COX subunit I gene expression was also induced after a blood meal in the *Ixodes ricinus* gut, salivary gland and hemolymph [46].

The silencing of the five selected genes resulted in no reduction in bacterial loads during transmission feeding, except in salivary glands from dsDYN + SYN-injected ticks. In contrast with our results, a previous study demonstrated that the knockdown of dsGST in *D. variabilis* resulted in a reduction of the *A. marginale* infection in the gut and salivary glands [47]. These differences in outcome could be associated with the use of different *A. marginale* strains and/or different species of ixodid ticks.

In addition to the impact of the gene knockdown on tick infection, we were interested in evaluating the transmission of *A. marginale* to calves after tick gene silencing. Interestingly, only the ticks injected with dsCOXIII failed to transmit *A. marginale* as determined by serology, PCR and Giemsa stained blood smears in

### Figure 4. Impact of tick gene knockdown on *A. marginale* transmission to calves.

(A) Detection of *A. marginale* in the blood of calves used as hosts for infected tick feeding at multiple time points. Recipient calves (represented by numbers) received specific dsRNA injected ticks. Giemsa blood smear: microscopic examination of a minimum of 50 high-power fields. MSP5n PCR: amplification of *msp5* by nPCR. MSP5c ELISA: Competitive inhibition enzyme-linked immunosorbent assay for MSP5 protein detection. nt: not tested. (B) Visualization of MSP5 nPCR products from calves blood (panel A) from trials 1 and 2 at the end of the 12th week by agarose gel electrophoresis stained with SYBR Safe (Invitrogen). Control +: blood from an *Anaplasma*-infected calf. Control -: without template DNA.
two distinct trials. The monitoring of *Anaplasma* in the blood of the calves was performed for up to 12 weeks after tick feeding without finding any sign of infection. Previous studies using similar experimental conditions demonstrated that 25–30 days post-tick feeding is the period that elapses between the ingress of the etiologic agent and the emergence of the earliest detectable forms of such agents [48,49]. The absence of *Anaplasma* infection in the calves that received dsCOXIII ticks could not be due to animal age because animals of any age can be infected, and the severity of disease is age-dependent [48]. We propose two biological explanations for the lack of *A. marginale* transmission by ticks injected with dsCOXIII: (i) COXIII knockdown interfered with the release of *A. marginale* into saliva either completely or below the transmission threshold and/or (ii) the gene knockdown affected *A. marginale* viability. Experiments are being conducted to elucidate how COXIII knockdown affects *A. marginale* transmission to calves.

Cytochrome c oxidase (COX) or complex IV is the terminal enzyme of the mitochondrial electron transport chain. The subunit III of COX (COXIII) is encoded by the mitochondrial genome and, with COXI and COXII, constitutes the COX catalytic core [50]. COX drives electrons that flows from cytochrome c to molecular oxygen and promotes the proton pump to the intermembrane space that is used to produce ATP. In addition, mitochondrial oxidative metabolism is also a major source of cellular ROS. One consequence of a poorly functioning COX is a reduction in ATP generation. The release of *Anaplasma* into tick saliva may be compromised because it has been postulated that *A. marginale* exit from tick cells by fusing the colony with the cell membrane [51], which requires ATP. Another effect of COX malfunction is the large production of ROS, resulting in cellular damage [52]. In yeast cells, partially assembled cytochrome oxidase produces increased ROS levels that block proliferation of the cells [53]. Such increased levels of ROS due to a dysfunctional cytochrome c oxidase could affect *A. marginale* viability and consequently its transmission to the calves.

Previous studies have demonstrated that the knockdown of several tick genes, including subolesin [13,47,54,55], GST [47], salivary selenoprotein M [47], H+ transporting lysosomal vacuolar proton pump [47] and varisin [56], reduce *A. marginale* levels in the gut and salivary gland. However, none of these studies evaluated the effect of gene silencing on bacterial transmission to naïve calves. The data presented here showed clearly that COXIII knockdown provoked a failure of *A. marginale* transmission to calves even while it did not affect the bacterial load in the ticks. This is the first report demonstrating that knockdown of a tick gene is associated with a miscarriage of *A. marginale* transmission.

**Supporting Information**

**Table S1** Primer sets for RT-qPCR and dsRNA.

**Table S2** Hyperlinked excel spreadsheet with assembled up and down contigs from SSH libraries and details of the sequence match.

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**Author Contributions**

Conceived and designed the experiments: TDB MWU EE GAS RGB SD. Wrote the paper: TDB MWU SD. Performed the experiments: TDB MWU KER. Analyzed the data: TDB MWU KER GRGB RGB RD. Wrote the paper: TDB MWU SD. Performed the bioinformatics analysis: GRCB. Performed the statistical analysis: SNW.

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