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Molecular and biological characterization of the *Amblyomma americanum* organic anion transporter polypeptide

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SUMMARY

The organic anion transporting polypeptides (Oatps in rodents and other organism; OATPs in human) are Na^+-independent transporters that shuttle a wide range of endogenous and xenobotic amphipathic compounds across plasma membranes. We previously discovered an *Amblyomma americanum* tick (*Aam*) Oatp cDNA among genes that were upregulated or induced in ticks that were stimulated to start feeding. In this study, we have characterized a 2860 bp full-length cDNA that encode a 724 amino acid putative protein. Bioinformatics and hydropathy analyses revealed that, in addition to the kazal-type serine proteinase inhibitor motif, *Aam*Oatp possess typical features that characterize the Oatp/OATP protein family, including 12 transmembrane (TM) domains, the consensus amino acid motif D-X-RW-(I,V)-GAWW-X-G-(F,L)-L and 11 consensus cysteine residues in the large extracellular domain between TM9 and TM10. *Aam*Oatp is constitutively and ubiquitously expressed, as determined by RT-PCR amplification of the transcript, in all organs of ticks that fed for 1–7 days. Analysis of the normalized transcript abundance revealed that from days 1 to 5 of feeding, *Aam*Oatp mRNA expression in the midgut (MG) was 60–80-fold higher than levels found in the salivary gland (SG), ovary (OV) and carcass (CA). By contrast, by day 7 of feeding, the *Aam*Oatp mRNA was 60–80-fold more strongly expressed in the OV than in the SG, MG and CA. These data strongly indicate that changing physiological needs during the tick feeding process influences transcriptional regulation of *Aam*Oatp. Our data also show that RNAi-mediated suppression of the *Aam*Oatp caused ticks to obtain smaller blood meals, which consequently resulted in ticks laying fewer eggs. The results are discussed in the context of *Aam*Oatp as a potential pharmacological or anti-tick vaccine target.

Key words: organic anion transporting polypeptide, RNAi gene silencing, *Amblyomma americanum*, tick feeding.

INTRODUCTION

Ticks are among the most successful globally distributed disease vectors with an extensive veterinary and public health impact (Sonenshine, 1993). They transmit the highest number of pathogens of all known vector arthropods and are considered second only to mosquitoes in terms of the importance of the vectored human pathogens they carry (Sonenshine, 1993). In the livestock industry, annual monetary losses due to ticks and tick-borne diseases amount to millions of dollars (Jongejan and Uilenberg, 2004; Guerrero et al., 2006). The control of ticks and tick-borne diseases has primarily been achieved by acaricide application, which has led to the selection of resistant tick populations, and contamination of the environment and of animal products (dela Fuente and Kocan, 2006). To forestall the possibility of failed tick control programs, development of a new generation of tick control methods has been advocated (Willadsen, 1990). The limiting step to this has been the lack of effective targets that, if disrupted alone or in a cocktail with others, will impede tick feeding success and/or pathogen acquisition and transmission. Our laboratory is investigating the molecular basis of how ticks begin to feed as a means of discovering new targets for rational design of novel tick control methods. We recently identified a putative *Amblyomma americanum* tick organic anion transporter polypeptide among genes that are induced or differentially upregulated in ticks that have attained appetence and/or been exposed to feeding stimuli (Mulenga et al., 2007).

The organic anion transporting polypeptides [abbreviated as 'Oatp' in non-human organisms and 'OATP' in humans (Meier-Abt et al., 2005; Konig et al., 2006; Hagenbuch and Meir, 2004)] are gene products of the recently recognized solute carrier gene superfamily [non-human, *Slco*; human, *SLCO* (Meier-Abt et al., 2005)]. Oatp/OATP proteins have been identified in a broad range of organisms, including rodents, humans, chickens, zebrafish, frog and insects (Hagenbuch and Meier, 2004). They are multi-specific Na^+-independent transmembrane transporters that are found in a wide spectrum of endogenous and xenobiotic amphipathic substrates, such as bile acids, bilirubin, thyroid hormones, prostanoids (prostaglandins and leukotrienes), neutral steroids (such as cardiac glycosides, ouabain and digoxin) and numerous other drugs (Meier-Abt et al., 2004; Konig et al., 2006; Niemi, 2007; Pizzagalli et al., 2002). In humans, one of the important physiological functions of the Oatp/OATP proteins is their role as components of the detoxifying mechanism of the body (Kasuhara and Sugiyama, 2005; Naud et al., 2007). Liver-, kidney- or the blood–brain barrier-expressed Oatp/OATP proteins mediate uptake of potentially toxic amphipathic substances from blood circulation into cells of these organs, where they are inactivated and then excreted as harmless by-products (Kasuhara and Sugiyma, 2005; Naud et al., 2007). Similarly, a recent study (Torrie et al., 2004) suggests that Oatp proteins may be part of the detoxification mechanism in insects. These authors recently explained the paradox of why ouabain, a cardiac glycoside and potent inhibitor of Na^+, K^+ and ATPase, fails

to inhibit fluid secretion by Malpighian tubules in many insects, by linking its clearance from the basolateral folding of *Drosophila* renal tubules to expression of *Oatp58Db*.

The aim of this study was to characterize the full sequence of *Aam*Oatp and validate its importance in tick feeding. We have provided evidence showing that *Aam*Oatp is constitutively and ubiquitously expressed, and that changing physiological needs during the tick feeding process influence its transcription patterns. Our data also show that RNAi mediated suppression of the *Aam*Oatp caused ticks to obtain smaller blood meals and consequently to lay fewer eggs.

MATERIALS AND METHODS

Tick dissections, total RNA and mRNA extraction

Amblyomma americanum L. ticks used in this study were obtained from a colony that is maintained on cattle in our department. Dissections of partially fed ticks were routinely carried out as previously published (Mulenga et al., 2003). Ticks partially fed for 24, 48, 72, 96, 120 and 168 hours were detached and rinsed in 70% ethanol and then washed in sterile 0.1% diethyl pyrocarbonate (DEPC) treated water. Washed ticks were placed onto a glass slide with a pair of soft tissue forceps and their edges were trimmed off using a sharp and sterile razor blade. After trimming, the tick was transferred onto the concavity of a hanging drop slide filled with 200 µl of DEPC treated water. Under a dissection microscope, the dorsal cuticle flap was lifted and tick organs: salivary glands (SG), midgut (MG) and ovary (OV) were teased out using an 18-gauge needle and a soft tissue forceps. All dissected tissues, including the carcass (CA), which represents the tick remnant after removal of SG, MG and OV, were homogenized in Trizol (Invitrogen, Carlsbad, CA, USA), the total RNA extraction reagent, and then stored at –80°C until needed. Prior to RNA extraction, whole ticks were rinsed in 70% ethanol, air dried, pulverized in liquid nitrogen and immediately transferred to the Trizol reagent. Total RNA was isolated by isopropanol precipitation and reconstituted in RNAse free water. Subsequently, mRNA was isolated from purified total RNA of unfed ticks using the Poly (A) Purist kit (Ambion, Austin, TX, USA) according to instructions provided by the manufacturer.

Cloning of full-length *A. americanum* (*Aam*) organic anion transporter polypeptide (Oatp)

To clone the full-length *Aam*Oatp cDNA, 2 µg of mRNA was used to synthesize the 3′ and 5′ adapter-linked cDNA templates according to instructions in the RLM rapid amplification of cDNA ends (RACE) protocol (Ambion). 3′ and 5′ RACE PCR primers that were synthesized based on a previously cloned *Aam*OATP cDNA fragment (Mulenga et al., 2007). Subsequently, these primers were used to amplify and clone the full-length cDNA using high-fidelity Taq polymerase (Applied Biosystems, Foster City, CA, USA) for long-distance PCR. PCR amplicons were routinely cloned in the TOPO PCR4 plasmid (Invitrogen) and sequencing was accomplished using the Bigdye terminator (Applied Biosystems), M13 forward and reverse primers, and internal primers were necessary. DNA sequence analysis was routinely carried out using the VectorNTI software (Invitrogen academic license).

Identification of *Aam*Oatp homologs in *Ixodes scapularis* genome

To identify homologs in the *Ixodes scapularis* Say genome, the FASTA3 software version 3.4 (Pearson and Lipman, 1988) was used to perform a local BLASTX scan of supercontig downloads from vector base (Lawson et al., 2007) (http://iscapularis.vectorbase.org/GetData/Downloads) using the deduced *Aam*Oatp as query. To identify coding exons, the retrieved *I. scapularis* supercontigs were scanned against *Drosophila* sequences using BLASTX analysis. Subsequently, best matches from the BLASTX scan and the *Aam*Oatp deduced protein were aligned with *I. scapularis* supercontigs using GenomeScan (Burge and Karlin, 1998) or the NetGene2 (Hebsgaard et al., 1996) servers to delineate introns and exons regions boundaries. The predicted exon regions marked by the ‘AT’ acceptor and ‘GT’ donor splice sites were manually assembled and translated using VectorNTI (Invitrogen).

Multiple sequence alignments and phylogeny analysis

Identity and similarity comparison with other sequences was accomplished using the BLASTX analysis at the NCBI. the levels of identical amino acids between *Aam*Oatp and other Oatp/OATP sequences were determined by pairwise alignment analyses using the VectorNTI software. The guide phylogeny tree was constructed from the dataset of Oatp/OATP protein sequence download from GenBank and four tick Oatp sequences (one from this study and three from the *I. scapularis* genome project) using the ‘neighbor joining’ method. Specifications were set for bootstrap values at 1000 replications with gaps proportionately distributed and correction for distance set to a ‘Poisson distribution’.

Tissue distribution analyses

To determine spatial and temporal expression profiles, DNAse-treated total RNA from SG, MG, OV and CA dissected from partially fed ticks (24, 48, 96, 120 and 168 h) was subjected to two-step semi-quantitative RT-PCR. Approximately 5 µg of total RNA was used to synthesize the first-strand cDNA template using the Verso cDNA kit (Thermo Fisher Scientific, Pittsburgh, PA, USA). 1 µl of the first strand cDNA was used as template in a PCR reaction with *Aam*Oatp specific primers. A 15 µl aliquot of the PCR product was electrophoresed on a 2% agarose gel containing 1 µg ethidium bromide.

To determine *Aam*Oatp mRNA abundance, densitograms of amplified PCR bands were determined using the web-based ImageJ image analyzer software (http://rsb.info.nih.gov/ij/). To correct for any differences resulting from variations between template concentrations, densities of detected PCR bands were normalized according to the following formula:

$$Y = V + V(H - X) / X,$$

where Y=normalized mRNA density, V=observed *Aam*Oatp PCR band density in individual tissues (MG, SG, OV and CA), H=highest tick actin PCR band density in tested tissues per time point and X=tissue (MG, SG, OV and CA) tick actin PCR band density.

RNA interference (RNAi) gene silencing

RNAi gene silencing was carried out according to previously published methods (Nijhof et al., 2007; de la Fuente et al., 2006; Decrem et al., 2008; Hatta et al., 2007). Using the cloned *Aam*Oatp plasmid DNA as template and PCR primers (forward, 5′**TAA TAC GAC TCA CTA TAG GG**G GAC CCG GCA AGC ACT TGC TAG TT3′; reverse, 5′**TAA TAC GAC TCA CTA TAG GG**T GTT CTT GGA GAC CGC GTC CTC C3′**)** with added T7 promoter sequence (in bold), were used to amplify the template for *Aam*Oatp double-stranded RNA (dsRNA). For negative control dsRNA, the green fluorescent protein (GFP) PCR primers with added T7 promoter sequence (forward, 5′**TAA TAC GAC TCA CTA TAG GG**TGGCCGCTTTACTTGTACAGC3′; reverse 5′**TAA TAC GAC TCA CTA TAG GG** ACCGGTCGCCACCATGGT3′) were

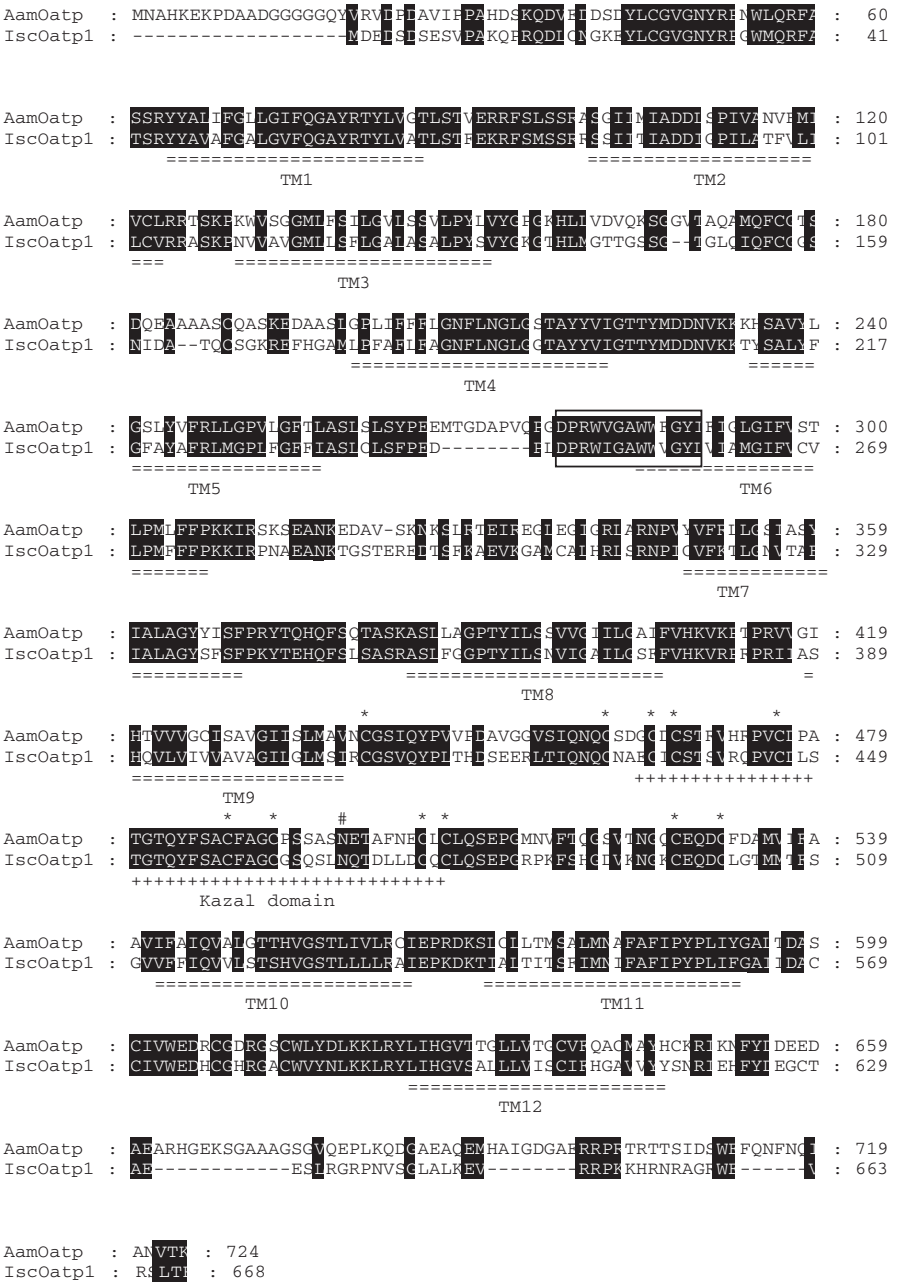

Fig. 1. Pairwise alignments of *Amblyomma americanum* (*Aam*) Oatp and *Ixodes scapularis* (*Isc*) Oatp1 amino acid sequences. As outlined in the Results section, *Isc*Oatp1 is one of the three full sequences that we have annotated from the *I. scapularis* genome. In our preliminary alignment, *Isc*Oatp2 and *Isc*Oatp3 showed less than 30% identity to *Aam*Oatp and they were excluded from this alignment. Similar and identical amino acid residues are highlighted in black. Transmembrane domains (TM1 to TM12) are double underlined by broken lines. The 11 conserved cysteine residues located between TM9 and TM10 are indicated by an asterisk. + indicates the putative N-glycosylation site. The Oatp/OATP protein family signature amino acid motif D-X-RW-(I,V)-GAWW-X-G-(F,L replaced by Y)-L (replaced by I) is boxed.

used to amplify the amplicon. Subsequently, dsRNA was synthesized *in vitro* using the MegaScript RNAi kit according to instructions provided by the manufacturer (Ambion). After purification of dsRNA, 20 unfed female ticks were microinjected with ~1 µl (~3 µg µl^{-1} in TE buffer, pH 8.0) of *Aam*Oatp or GFP dsRNA via the groove between the basis capituli and the scutum using half-inch 33-gauge needles attached to a 10 µl gastight syringe (Hamilton, Reno, NV, USA). Injected ticks were kept overnight at 22°C to observe any mortality resulting from injection injury. Subsequently *Aam*Oatp- and GFP-dsRNA-injected and non-injected control ticks (*N*=20) were put in cells that were secured on the back of the calf using livestock identification cement (Nasco, Fort Atkinson, WI). Ticks were allowed to feed until detachment.

To validate the specificity of RNAi-mediated silencing of *Aam*Oatp, three ticks per treatment (*Aam*Oatp-dsRNA and GFP-dsRNA-injected) were detached 48 h post-attachment. These ticks were individually processed for total RNA extraction. Total RNA was treated with RQ1 DNAse (Promega, Madison, WI, USA) to remove genomic DNA (gDNA) contamination and then subjected to two-step semi-quantitative RT-PCR. To forestall the possibility of re-amplifying the injected *Aam*Oatp-dsRNA during validation of *Aam*Oatp silencing, PCR primers (forward, 5′GACGAATTCGTG-CAGCGACGGCTGCGACTG3′; reverse, 5′CTGAAGCTTTCA-CTTGGTCACGTTGGCGAT3′) were targeted to cDNA regions flanking the domain that was used for dsRNA synthesis. Lack of amplification of the *Aam*Oatp transcript confirmed the silencing of *Aam*Oatp.

To assess the effect of silencing *Aam*Oatp on tick-feeding success, tick-feeding parameters, including numbers of attached ticks 24 h post-attachment, mortality, time of feeding to repletion, survival and engorgement masses (EM, measure of amount of blood imbibed) were evaluated. To assess the effects of *Aam*Oatp silencing on

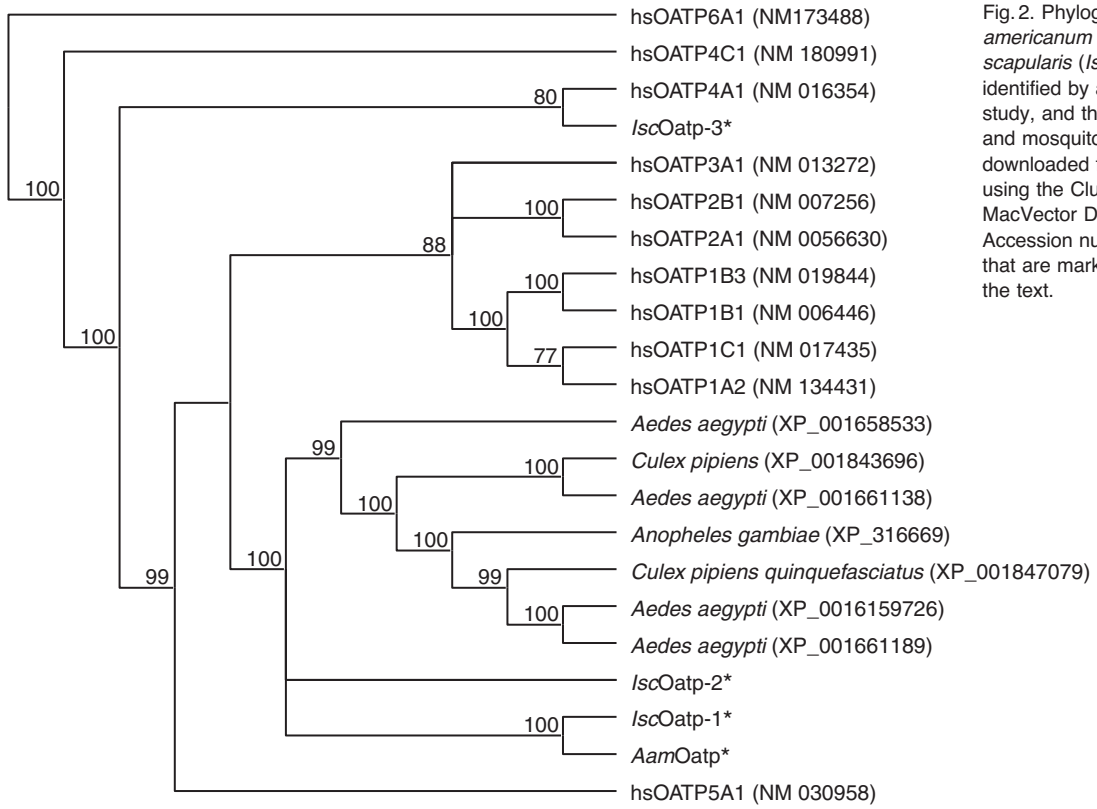

Fig. 2. Phylogenic analysis: the *Amblyomma americanum* (*Aam*) Oatp and three *Ixodes scapularis* (*Isc*) Oatp sequences that were identified by a third-party annotation in this study, and the indicated *Homo sapiens* (hs) and mosquito sequences that were downloaded from GenBank were aligned using the Clustal W algorithm contained in MacVector DNA sequence analysis software. Accession numbers of tick Oatp sequences that are marked with asterisks are given in the text.

fecundity, the egg mass conversion ratios (EMCR) were compared between treatment and control ticks. This was accomplished by incubating engorged ticks at 25°C for 20 days to allow them to lay eggs and then the egg masses were weighed. The EMCR was calculated by mass of the egg mass as a fraction of the EM. A web-based unpaired Student's *t*-test (GraphPad *t*-test calculator, www.graphpad.com/quickcalcs/ttest1.cfm) was used to test the statistical significance of the differences that were observed between *Aam*Oatp-dsRNA-injected ticks and control groups.

RESULTS

Putative *Aam*Oatp protein has typical features of the Oatp/OATP protein family

Rapid amplification of cDNA ends was successfully used to clone a 2860 bp full-length cDNA (GenBank accession number FJ206228) that encode a 724 amino acid protein (Fig. 1). Provisional identification of *Aam*Oatp as a member of the Oatp/OATP protein family (Hagenbuch and Meier, 2004) was based on its similarity to previously characterized proteins (data not shown). When scanned against known protein entries in GenBank, the top 20 best matches to *Aam*Oatp were arthropod, including fruit flies (*Drosophila* spp), the jewel wasp (*Nansonia vetripennis*), the red flower beetle (*Tribolium castaneum*), mosquitoes (*Aedes* and *Anopheles* spp) and honeybee (*Apis melifera*) (data not shown). Hydropathy and bioinformatics analyses, revealed that *Aam*Oatp contains the Kazal type serine proteinase inhibitor domain in addition to all typical features of Oatp/OATP proteins: 12 transmembrane (TM) domains, a 13 amino acid signature motif, D-X-RW-(I,V)-GAWW-X-G-(F,L replaced by Y)-L (replaced by I) and a large extracellular region (ER) between the ninth and tenth TM domains (Fig. 1). Consistent with other sequences, 11 consensus cysteine residues that are present in the large ER of all Oatp/OATP proteins (Meier-Abt et al., 2005; Hagenbuch and Meier, 2003; Hagenbuch and Meier, 2004) are 100% conserved in *Aam*Oatp (Fig. 1). Visual analysis of the *Aam*Oatp deduced amino acid sequence revealed that, unlike most known vertebrate Oatp/OATP protein sequences, which posses two putative N-glycosylation sites, in the second and fifth ER (Meier-Abt et al., 2005; Hagenbuch and Meier, 2004) the *Aam*Oatp protein posses one putative N-glycosylation site in its fifth ER (Fig. 1). It is interesting that the *Aam*Oatp protein also contains two additional putative N-glycosylation sites. However, these may not be functional as they are located within the second and fifth intracellular domains (Fig. 1).

When scanned against supercontigs of the *I. scapularis* genome, *Aam*Oatp produced hits to eight supercontigs (not shown), which were determined to contain 11 different *I. scapularis* (*Isc*) Oatp sequences. Because of gaps in supercontig sequences, eight of the 11 *Isc*Oatp sequences are partial and the rest could be assembled into complete open reading frames. Among the three complete sequences, *Aam*Oatp show 50/67% identity/similarity to *Isc*Oatp-1 (Fig. 1), 26/41% to *Isc*Oatp-2 and 21/42% to *Isc*Oatp-3 (not shown). Consistent with our multiple sequence alignment data in Fig. 1, phylogenetic analysis revealed that *Aam*Oatp sequence was closely related to *Isc*Oatp-1 (Fig. 2). From the phylogeny tree in Fig. 2, it is interesting to note that *Isc*Oatp-2 is distantly related to other arthropod sequences, but it is closely related to human OATP4A1. At the time of preparing this manuscript, the annotation of the *I. scapularis* genome had not been completed. Thus accession numbers of the primary sequences that were used to assemble ORFs, ABJB010623253.1, ABJB010665419.1 and ABJB010386346.1 for *Isc*Oatp-1, ABJB011035923.1 for *Isc*Oatp-2 and ABJB010326688.1 for *Isc*Oatp-3 are indicated.

Changing physiological needs during tick feeding influences transcriptional regulation of *Aam*Oatp

To assess temporal and spatial expression profiles of *Aam*Oatp mRNA in tick organs (SG, MG, OV and CA) during tick feeding,

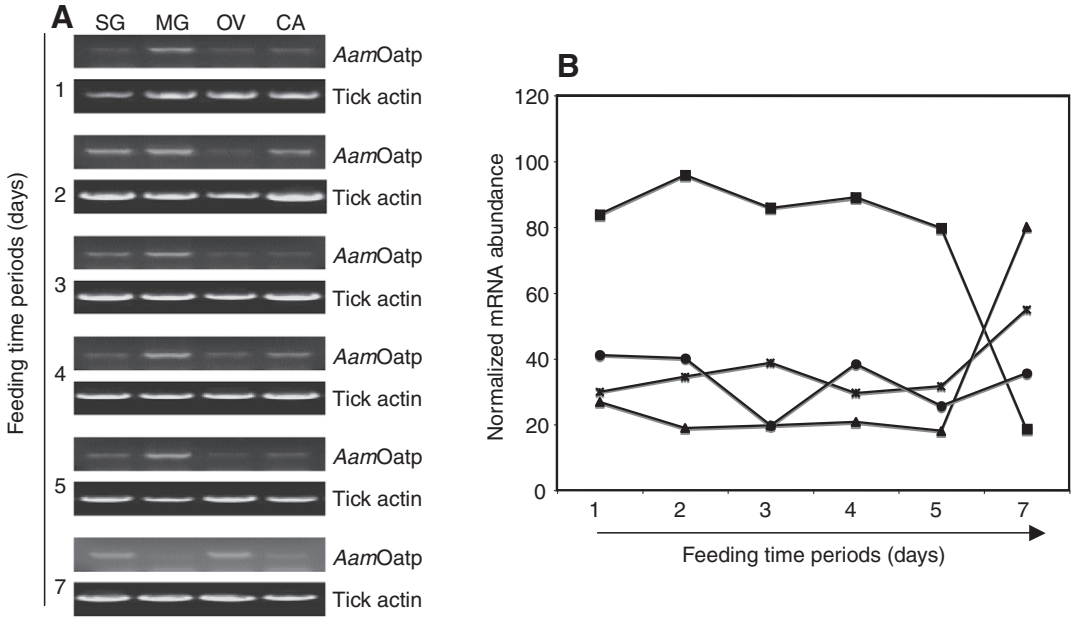

Fig. 3. Temporal and spatial mRNA expression profile of *Aam*Oatp during tick feeding. (A) DNAse-treated total RNA of salivary gland (SG), midgut (MG), ovary (OV) and carcass (CA, tick remnants after removal of SG, MG and OV) dissected from ticks that were partially fed for 1–7 days were subjected to two-step semi-quantitative RT-PCR. (B) Densities of *Aam*Oatp and tick actin PCR bands were determined using the web-based ImageJ program, as described in the Materials and methods. Band densities representing mRNA levels in tick organs were normalized according to the formula $Y=V+V(H–X)/X$, where Y=normalized mRNA density, V=observed *Aam*Oatp PCR band density in individual tissues (MG, SG, OV and CA), H=highest tick actin PCR band density in tested tissues per time point, and X=tissue (MG, SG, OV and CA) tick actin PCR band density. The crosses, squares, triangles and circles indicate SG, MG, OV and CA, respectively.

total RNA of organs from ticks that fed from 1–7 days, was subjected to semi-quantitative RT-PCR analysis (Fig. 3). Results summarized in Fig. 3A show that *Aam*Oatp mRNA is ubiquitously expressed, as revealed by its being amplified from cDNA of all tick organs. Analysis of the normalized transcript abundance shown in Fig. 3B revealed that from day 1 to 5 of feeding, *Aam*Oatp mRNA in the MG was ~60–80-fold higher than levels found in SG, OV and CA. By contrast, by day 7 of feeding, the *Aam*Oatp mRNA was 60–80-fold more strongly expressed in the OV than the SG, MG and CA (Fig. 3B).

Silencing of *Aam*Oatp caused ticks to obtain significantly smaller blood meals and diminish their egg mass conversion ratio

Qualitative two-step RT-PCR expression analysis summarized in Fig. 4 was used to validate the RNAi-mediated silencing of *Aam*Oatp mRNA. On the basis of detectable PCR bands amplified by 40 PCR cycles, complete silencing of *Aam*Oatp was confirmed in two out of the three ticks tested (Fig. 4). In order to assess the effect of silencing on tick-feeding efficiency, feeding parameters were compared between ticks that were injected with *Aam*Oatp-dsRNA and those injected with GFP-dsRNA. Silencing of *Aam*Oatp did not affect the ability of ticks to attach onto host skin, initiate and continue to feed (results not shown). We also found that silencing of *Aam*Oatp did not cause any mortality (results not shown).

To quantify the amount of blood taken in by a tick, we determined engorgement masses (EM, mass of ticks upon naturally detaching from the host, representatives are shown in Fig. 5A). The assumption was that the EMs represented the amount of blood taken in by a tick. EMs summarized in Fig. 5B were 532–940 mg (N=15, mean ± s.e.m.=730±37.58 mg) for non-injected controls, 437–774 mg (N=10, mean ± s.e.m.=620±31.95 mg) for GFP-dsRNA-injected controls, 283–647 mg (N=10, mean ± s.e.m.=482±31.21) for *Aam*Oatp-dsRNA-injected ticks. The unpaired Student's t-test comparison of mean EMs showed that blood meals obtained by *Aam*Oatp-dsRNA-injected ticks were significantly smaller than those obtained by both non-injected (P<0.0001, two-tailed test) and GFP-dsRNA-injected (P=0.0073, two-tailed test) controls ticks. Importantly, blood meals obtained by GFP-dsRNA-injected control ticks were also statistically significantly smaller than those obtained in the non-injected controls (P=0.0479, two-tailed test).

In order to assess the effect of silencing *Aam*Oatp on fecundity, we compared the ability of ticks to convert their blood meal into the egg mass. As was to be expected, *Aam*Oatp-dsRNA-injected

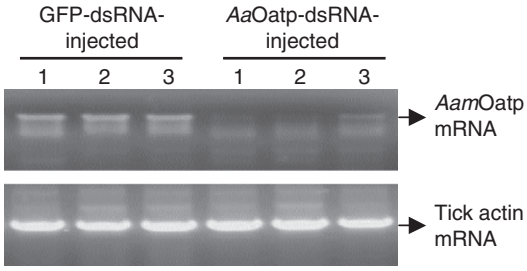

Fig. 4. Validation of RNAi-mediated silencing of *Aam*Oatp. three ticks that were injected with GFP-dsRNA or *Aam*Oatp-dsRNA were manually detached at 48 h post-attachment. These ticks were individually processed for total RNA extraction. Extracted total RNA was DNAse treated and then subjected to two-step RT-PCR. PCR products were electrophoresed on a 2% agarose gel containing 1 μg ml^{-1} of ethidium bromide. The numbers represent individually treated ticks.

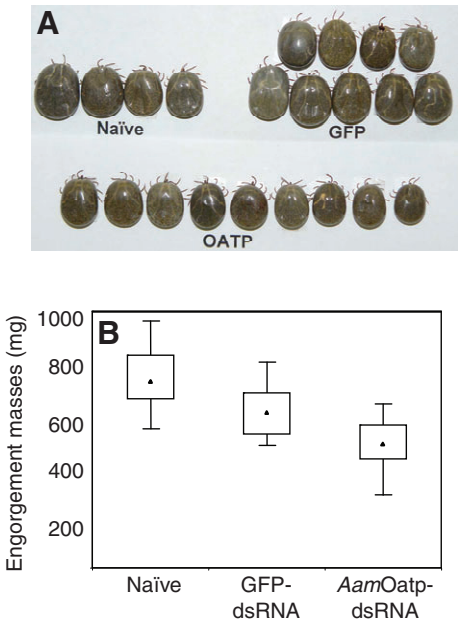

Fig. 5. Effects of RNAi-mediated silencing of *Aam*Oatp on *Amblyomma americanum* tick-feeding success: the blood meal size. Ticks injected with *Aam*Oatp-dsRNA, control ticks, uninjected ticks and those injected with GFP-dsRNA were allowed to feed to repletion on cattle. After feeding to repletion (representatives are shown in A), engorgement masses (EMs) of ticks were determined. Summarized in B are EMs of 532–940 mg (N=15, mean=730) for uninjected controls, 437–774 mg (N=10, mean=620) for GFP-dsRNA-injected controls and 283–647 mg (N=10, mean=484) for *Aam*Oatp-dsRNA-injected ticks. The mean EM of *Aam*Oatp-dsRNA-injected ticks was significantly lower than that of the uninjected (P<0.0001, two-tailed test) and GFP-dsRNA-injected (P=0.0073, two-tailed test) control ticks. The box plots in this figure were developed with Microsoft Excel using mean, median, minimum and maximum, the first and third quartile data points. The bars in the graph represent maximum and minimum data points, as indicated in the text.

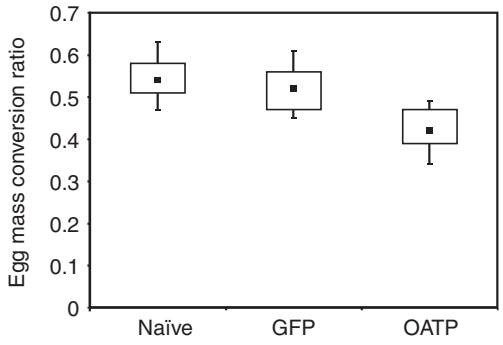

Fig. 6. Effects of silencing *Aam*Oatp on *Amblyomma americanum* fecundity. After feeding to repletion, ticks were incubated at 25°C for 20 days to lay eggs. Egg masses were weighed then divided by engorgement masses (EMs) to calculate the egg mass conversion ratio (EMCR). The significance of difference between the mean EMCRs of *Aam*Oatp-dsRNA (mean=0.423) and control ticks non-injected (mean=0.541, P<0.0001) and those injected with GFP-dsRNA (mean=0.515, P=0.0008) were determined using the unpaired Student's t-test. The bars in this graph represent maximum and minimum data points, as indicated in the text.

ticks, which had obtained smaller blood meals (Fig. 5B), laid apparently fewer eggs (mean ± s.e.m.=205±14.95) than both the non-injected (mean ± s.e.m.=400±21.21) and the GFP-dsRNA-injected (mean ± s.e.m.=318±18.43) control groups. Comparison of EMCRs summarized in Fig. 6 show that silencing of *Aam*Oatp caused a significant reduction in the efficiency of the tick to convert blood meal nutrients into eggs (P<0.0001 versus non-injected controls) (P=0.0008, *versus* GFP-dsRNA-injected controls). By contrast, EMCR of GSP-dsRNA-injected control ticks did not statistically differ from the non-injected control (P=0.2199), although they laid significantly fewer eggs.

DISCUSSION

Several Oatp/OATP proteins have been identified in a broad range of organisms, including rodents, humans, chickens, zebrafish, frog and insects (Hagenbuch and Meier, 2004). This study describes the first report on biological and bioinformatics characterization of an Oatp protein from a blood-feeding organism. Based on data in this study, we are unable to determine conclusively whether or not the *Aam*Oatp protein is functional. However, based on our bioinformatics analyses, we believe that the *Aam*Oatp protein is likely to be a functional transporter because of the high conservation of sequence structures that are thought to be important for the transport function of Oatp/OATP proteins (Meier-Abt et al., 2005; Hänggi et al., 2006). In a recent study, Hanggi et al. (Hanggi et al., 2006) showed a single mutation among the 11 consensus cysteine residues in the large ER between TM9 and TM10 was enough to destroy the transport function of the OATP2B1 protein. Given that these cysteine residues are 100% conserved in the *Aam*Oatp, it is a good indicator that this protein is likely to be functional.

In humans, mice and rats, at least 39 Oatp/OATP proteins were described by 2005 (Meier-Abt et al., 2005). Given that the *A. americanum* genome has not been sequenced, the full repertoire of Oatp proteins in this tick will remain unknown. However, based on our analysis of supercontigs of the *I. scapularis* genome, ticks may encode at least 11 different Oatp proteins. In a recent study, Hagenbuch and Meir (Hagenbuch and Meir, 2004) suggested a new classification system of Oatp/OATP proteins, where sequences that show ≥40% identity belonged into the same family and those showing ≥60% identity being further classified into a subfamily. On the basis of this classification system, the four tick Oatp proteins in this study (three *Isc*Oatp and the *Aam*Oatp proteins) may be classified into at least three families: *Aam*Oatp and *Isc*Oatp-1, which are 56% identical belonging to the same family; with *Isc*Oatp-2 and -3, which showed <40% identity to any of the sequences, belonging to distinct families. From our phylogenetic analysis data, it was interesting that *Isc*Oatp-3 was more closely related to the human OATP4A1 protein than to other arthropod sequences. OATP4A1 is a known transporter of prostaglandin, among other substrates (Gao et al., 2005). Ticks do synthesize prostaglandins (PGs), which are thought to play a role in facilitation of tick–host interactions (Bowman et al., 1996). The *in vivo* transport of tick PGs has not been studied. It will be interesting to investigate whether *Isc*Oatp-3, similar to OATP4A1, is also involved in transport of PGs.

An important component of this study was to gain insight on the potential of *Aam*Oatp as a druggable or an anti-tick vaccine target. Our RNAi data suggest that *Aam*Oatp represents an important anti-tick target, as its silencing caused ticks to obtain significantly smaller blood meals. Although, silencing of *Aam*Oatp did not prevent ticks from attaching and starting to feed, these findings suggest that *Aam*Oatp may play a role in the processing of host blood meal. It is noteworthy that, although silencing of *Aam*Oatp did not prevent

egg laying, it reduced the ability of these ticks to convert their blood meals into eggs. A potential limitation to our approach is that we could not account for the actions of the pre-existing *Aam*Oatp protein prior to silencing. Our speculation is that, after silencing of the *Aam*Oatp mRNA, the pre-existing *Aam*Oatp protein will still be viable and thus the effects of silencing would not be immediate. This might explain the seemingly lack of impact of *Aam*Oatp silencing on early tick-feeding parameters, attachment onto host skin and beginning to feed.

Consistent with vertebrate Oatp/OATP genes that show multiple tissue expression patterns (St-Pierre et al., 2002; Gao et al., 2005; Choudhuri et al., 2003), our RT-PCR expression analyses data revealed that *Aam*Oatp mRNA is both constitutively and ubiquitously expressed, as it was it is expressed in all tested organs of ticks that fed for 1–7 days. Transcriptional regulation of tick genes has been shown in response to blood meal feeding, being either shut down or induced, up- or down regulated (Leboule et al., 2002a; Leboule et al., 2002b; Mulenga et al., 2001; Rudenko et al., 2005). In the case of *Aam*Oatp, the switch in expression pattern, from being predominant in the midgut during the first 5 days of feeding and the ovary, by day 7 of feeding suggest that *Aam*Oatp is transcriptionally regulated in response to changing physiological needs as tick feeding progresses. The first 5 days of feeding, when *Aam*Oatp is strongly expressed in the MG, correspond to the slow feeding phase (SFP) of the tick feeding cycle. This phase prepares the tick for the rapid feeding phase (RFP), during which it feeds to repletion (Sonenshine, 1993; Bowman et al., 1996; Reuben, 2007). Day 7 of tick feeding corresponds to the beginning of the RFP, and is when predominant expression of *Aam*Oatp switches to the ovary. Assuming that the *Aam*Oatp protein expression profiles are correlated with transcript production, we speculate that during the first 5 days of feeding, *Aam*Oatp is involved in transport of key nutrients (s) from host blood into tick hemolymph. Subsequently, as the tick feeds to repletion, *Aam*Oatp could be involved in transporting nutrients from hemolymph into the ovary for egg development. It is interesting that the thyroid hormone derivatives thyroxine and triidothyronine, which are transported across cell membranes by Oatp/OATP proteins (Hagenbuch and Meir, 2004; Pizzagalli et al., 2002), have been linked to embryo development in birds (Willson and McNabb, 1997). Whether or not this is the case in ticks is not known. However, if it were the case, it will be interesting to investigate whether the biological functions of the *Aam*Oatp protein are related to egg development. Whether or not the silencing of *Aam*Oatp reduces the efficiency of the tick to convert its blood meal to eggs is an indication of this possibility is not currently known.

Because of their ubiquitous distribution and their wide activity range as transporters of several drugs, the role of Oatp/OATP proteins in drug disposition, bio-distribution and absorption is emerging (van Montfoort et al., 2003; Faber et al., 2003; Mizuno et al., 2003; Gerloff, 2004; Niemi, 2007; Bachmakov et al., 2008). This study presents a framework for similar studies to be carried out in ticks. Although only a single study has linked the Oatp transmembrane transport function to the detoxification mechanisms in arthropods (Torrie et al., 2004), these proteins represent significant druggable or anti-tick vaccine targets as their blockade could prevent ticks from excreting important chemical compounds. As anti-tick vaccine targets, another attractive feature of these proteins is that they are mostly localized on cell surfaces (St-Pierre et al., 2002), which will make them accessible to host antibodies. The next phase of this research will be to characterize the transport functions of *Aam*Oatp and whether or they could be targeted for tick control.

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