Systems-Based Analysis of the *Sarcocystis neurona* Genome Identifies Pathways That Contribute to a Heteroxenous Life Cycle

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ABSTRACT *Sarcocystis neurona* is a member of the coccidia, a clade of single-celled parasites of medical and veterinary importance including *Eimeria*, *Sarcocystis*, *Neospora*, and *Toxoplasma*. Unlike *Eimeria*, a single-host enteric pathogen, *Sarcocystis*, *Neospora*, and *Toxoplasma* are two-host parasites that infect and produce infectious tissue cysts in a wide range of intermediate hosts. As a genus, *Sarcocystis* is one of the most successful protozoan parasites; all vertebrates, including birds, reptiles, fish, and mammals, are hosts to at least one *Sarcocystis* species. Here we sequenced *Sarcocystis neurona*, the causal agent of fatal equine protozoal myeloencephalitis. The *S. neurona* genome is 127 Mbp, more than twice the size of other sequenced coccidian genomes. Comparative analyses identified conservation of the invasion machinery among the coccidia. However, many dense-granule and rhoptry kinase genes, responsible for altering host effector pathways in *Toxoplasma* and *Neospora*, are absent from *S. neurona*. Further, *S. neurona* has a divergent repertoire of SRS proteins, previously implicated in tissue cyst formation in *Toxoplasma*. Systems-based analyses identified a series of metabolic innovations, including the ability to exploit alternative sources of energy. Finally, we present an *S. neurona* model detailing conserved molecular innovations that promote the transition from a purely enteric lifestyle (*Eimeria*) to a heteroxenous parasite capable of infecting a wide range of intermediate hosts.

IMPORTANCE *Sarcocystis neurona* is a member of the coccidia, a clade of single-celled apicomplexan parasites responsible for major economic and health care burdens worldwide. A cousin of *Plasmodium*, *Cryptosporidium*, *Theileria*, and *Eimeria*, *Sarcocystis* is one of the most successful parasitic genera; it is capable of infecting all vertebrates (fish, reptiles, birds, and mammals—including humans). The past decade has witnessed an increasing number of human outbreaks of clinical significance associated with acute sarcocystosis. Among coccidian species, *S. neurona* has a wide host range and causes fatal encephalitis in horses, marine mammals, and several other mammals. To provide insights into the transition from a purely enteric parasite (e.g., *Eimeria*) to one that forms tissue cysts (*Toxoplasma*), we present the first genome sequence of *S. neurona*. Comparisons with other coccidian genomes highlight the molecular innovations that drive its distinct life cycle strategies.

The coccidia are a large clade of protozoan parasites within the phylum Apicomplexa. In addition to a single definitive host species in which the parasite undergoes its sexual cycle, a subgroup of coccidia, the members of the family *Sarcocystidae* (*Sarcocystis*, *Toxoplasma*, and *Neospora*) have evolved the ability to infect a broad range of intermediate hosts (1, 2). To drive this transition, the members of the family *Sarcocystidae* produce infectious tissue cysts surrounded by glycosylated cyst walls. Different species and even strains exhibit distinct patterns of organ tropism, with *Toxoplasma* forming cysts in any organ, whereas *Sarcocystis* cysts are largely restricted to muscle. Ingestion of tissue cysts through predation or scavenging by the definitive host propagates the life cycle (e.g., felids for *Toxoplasma*, canids for *Neospora*, and humans for two *Sarcocystis* species) (3).
To survive and persist in their respective hosts, apicomplexan parasites have evolved a variety of molecular strategies. These include a group of specialized proteins that facilitate parasite entry, egress, and colonization, as well as molecular decoys that modulate host immune signaling (4, 5). The majority of these proteins localize to exocytic organelles (micronemes, rhoptries, and dense granules) that discharge in a highly coordinated program of invasion (5). For *Toxoplasma gondii*, initial host recognition and attachment are performed by members of the SAG1-related sequence (SRS) family. This is followed by secretion of the microneme (MIC) proteins that strengthen host cell attachment and result in the formation of a “moving junction” that provides the motive force required to penetrate the host cell. The moving junction is further controlled by a set of proteins known as rhoptry neck (RON) proteins that facilitate invasion (for a review, see reference 6). Subsequently, rhoptry (ROP) proteins and dense-granule (GRA) proteins are secreted into the host cytosol to interact with cellular targets to protect the (now) intracellular parasite from clearance. The parasite is further protected through encasement within a parasitophorous vacuole (PV) that is, interestingly, absent from the schizont form of *Sarcocystis*.

Recent genomic comparisons of *Toxoplasma*, *Neospora*, and *Hammondia* (the closest extant relative of *T. gondii*) have identified a series of ROP proteins whose expression targets host-specific immune signaling pathways. ROP5, ROP16, and ROP18 have all been shown to affect parasite virulence and contribute to host specialization in the mouse model (4, 5). Recently, an expanded repertoire of SRS proteins, previously implicated in host range expansion of *T. gondii*, were also identified in *Neospora* (7). Modeling of *T. gondii* metabolism also identified strain-specific differences in growth potential, establishing metabolism as an evolutionary factor capable of influencing host adaptation (8). To complement the recently generated *Eimeria* genome sequences and understand the transition from a purely enteric, monoxenous life cycle (e.g., *Eimeria*) to a heteroxenous one that includes the formation of tissue cysts, we sequenced the genome of *Sarcocystis neurona*.

Sarcocystosis, caused by parasites within the genus *Sarcocystis*, is typically asymptomatic but can be associated with myositis, diarrhea, or infection of the central nervous system (CNS). The genus is ancient (relative age, 246 to 500 million years based on small-subunit RNA sequences), diverse (more than 150 cataloged species), highly successful (all vertebrates are susceptible hosts, including fish, birds, reptiles, and mammals), and prevalent (cattle exhibit a 90% infection rate worldwide) (9). Interestingly, *Sarcocystis* species are not structurally similar; for example, *S. neurona* sporozoites, like *T. gondii*, lack the crystallloid body present in other coccidia, including *S. cruzi* of cattle (10). *Sarcocystis* species typically have a two-host predator-prey life cycle, with one host supporting asexual multiplication while the other acts as the definitive host, supporting a sexual cycle that results in sporocyst shedding in feces. Humans are definitive hosts of *S. hominis* and can be infected by *S. nesbitti*, with associated sequelae, including muscular sarcocystosis. Opossums are the definitive hosts of *S. hominis* (11), a species that has a broad intermediate-host range, including raccoons, cats, skunks, and more recently a variety of mustelids, pinnipeds, and cetaceans (12–15). *S. neurona* produces tissue cysts, typically in muscle and occasionally in the CNS (16, 17). Horses are considered aberrant hosts, in which the parasite typically multiplies as schizonts in the CNS but fails to encyst. Unabated destruction of neural tissue can be fatal to horses and many other hosts, and the disease was called equine protozoal myeloencephalitis before the etiologic protozoan *S. neurona* was identified and named in 1991 (2). With migration of opossums to the west coast of North America during the last century (14) the *S. neurona* host range expanded to cause epizootics in sea otters, harbor seals, and harbor porpoises (18). *S. neurona* is now being monitored for its potential as an emerging disease threat. Here, we sequenced and performed a systems-based analysis of the genome of type II *S. neurona* strain SO SN1, isolated from a southern sea otter that died of protozoal encephalitis (19), which represents the most common genotype infecting animals throughout the United States.

**RESULTS**

The *S. neurona* SO SN1 genome is more than twice the size of the *T. gondii* genome. Combining 7,020,033 reads from the 454 Life Sciences sequencing platform with 529,830,690 reads from the Illumina Hi-Seq sequencing platform, we generated 47,722 Mbp of sequence data from *S. neurona* SO SN1 DNA. By integrating a variety of assembly algorithms (see Materials and Methods), these data were assembled into 116 genomic scaffolds with a combined size of 127 Mbp, over twice the size of the *Neospora caninum* and *T. gondii* genomes (Table 1 is a summary of the genome statistics obtained). An additional 3.1 Mbp of sequence is encoded in 2,950 unscaffolded contigs (each greater than 500 bp in length). The assembly N50 value was 3,117,290 bp, with a maximum scaffold length of 9,217,112 bp. To help annotation efforts, we generated an additional 59,622,019 reads from *S. neurona* RNA. From these data, we predict a complement of 7,093 genes, 5,853 of which are supported by RNA-Seq evidence (see Table S1 in the supplemental material). The number of genes predicted is comparable to that of the related coccidia *T. gondii* and *Eimeria tenella*. Comparisons of gene orderings reveal blocks of syntenic relationships between homologous genes in *S. neurona* and *T. gondii*, with the longest such block aligning 43 genes on scaffold 1 of *S. neurona* and chromosome IX of *T. gondii* (Fig. 1A). Chromosome-wide synteny was not observed, suggesting a significant level of genome rearrangement between *S. neurona* and *T. gondii*. A more detailed view of the largest syntenic region reveals the extent of gene order preservation but also reveals differences in the structures of individual genes (Fig. 1B).

Given similarities in gene numbers and exon lengths, we next determined the source of the additional sequence associated with
Architecture and syntenic relationships of the *S. neurona* genome. (A) Circos representations (50) of syntenic relationships between the genomes of *S. neurona* and *T. gondii* ME49. The inner circle shows syntenic relationships among the 10 largest *S. neurona* genomic scaffolds (maximum size, 9.2 Mb; minimum size, 3.5 Mb) and the 14 chromosomes of *T. gondii*. Bandwidths indicate alignment length, and colors represent the *S. neurona* scaffold of origin for the gene clusters. Grey circles indicate the largest regions of genomic synteny between *S. neurona* scaffold SO SN1 and *T. gondii* chromosome 9. The outer circles show a (Continued)
families was almost exactly 2:1, the statistically expected rate of transitions in alignments of repetitive sequences to DNA repeats. Supporting this view, we found that the ratio of transversions to transitions, which mediates. The most abundant family of DNA elements belonged to the CACTA-Mirage-Chapaev family of transposons, although a few were composed of consensus sequences with lengths show that S. neurona possesses a similar number of exons per gene (5.50 in T. gondii, 5.93 in N. caninum, and 5.44 in S. neurona) but that the average length of the introns in S. neurona (1,437.5 bp) is roughly triple that of T. gondii and N. caninum (497.5 and 465.2 bp, respectively). Further, comparisons of intronic and intergenic regions reveal these to be larger in S. neurona (8,495 ± 222 bp) than in T. gondii and E. tenella (2,381 ± 72 and 2,934 ± 268 bp, respectively). To identify factors responsible for the increased intra- and intergenic region sizes, we performed a systematic analysis of repetitive regions across representative apicomplexan genomes with the software tool RepeatModeler (20). This analysis revealed that the S. neurona genome is rich in repeats largely associated with long interspersed nucleotide element (LINE) and DNA element sequences (class I and II transposons, respectively). Mapping of the repeats to scaffolds revealed that many of the repeats are associated with genes (Fig. 1B). Further comparisons of introns, exons, and intergenic regions showed clear differences in the repeat type based on the genomic context (Fig. 1C). DNA element-type repeats were enriched in intronic regions and virtually absent from exons, suggesting evolutionary pressure against the integration of DNA elements within coding regions. Conversely, LINE-like repeats were equally distributed across exons, introns, and intergenic regions. In total, 31 Mb of the S. neurona genome had repetitive sequences, compared to 17.9 Mbp of the E. tenella genome and 2.5 Mbp of the T. gondii genome (Fig. 2A).

S. neurona displays a diverse set of repetitive elements. The repetitive sequences present in S. neurona are extraordinarily diverse, with 203 families of repeats discovered with RepeatModeler, compared to 101 families in E. tenella and 5 in Plasmodium falciparum. The majority of simple repeats within the S. neurona genome belong to more diverse families, unlike other apicomplexan parasites, where simple repeats are largely composed of short repeats (e.g., CAGn in E. tenella [21]). For example, 33 simple repeat families in S. neurona were composed of consensus sequences with an average length of 287 bp. The average length of the simple repeats was 105 bp in S. neurona, compared to 48 and 68 bp in P. falciparum and E. tenella, respectively.

Type II transposons, or DNA elements with 64,732 members, represent the largest family of repeats present in S. neurona, totaling 14.6 Mbp (11.5%) of the genome, considerably more than in E. tenella (Fig. 2A). All of the DNA elements identified belong to the “cut-and-paste” families of transposons, which propagate through genomes through excision and insertion of DNA intermediates. The most abundant family of DNA elements belonged to the CACTA-Mirage-Chapaev family of transposons, although a minority of Mutator-like elements was also identified. Active DNA transposons contain transposase genes; however, we were unable to detect any such gene within the S. neurona genome, suggesting that these DNA elements are ancient and degraded. Supporting this view, we found that the ratio of transversions to transitions in alignments of repetitive sequences to DNA repeat families was almost exactly 2:1, the statistically expected rate of mutation in the absence of evolutionary pressure.

Given the relative lack of repeats in the T. gondii genome, we explored whether the repeats identified in S. neurona are less active than those identified in E. tenella. When repeats are active, it is possible to identify clades of repeats with significant sequence similarity. Pairwise sequence alignments of members of five families of repeats (Fig. 2B) were highly divergent, indicating that the LINEs and DNA elements are no longer active in S. neurona. Further, the LINEs in S. neurona are more diverse and therefore likely to be more ancient than those in E. tenella. Interestingly, E. tenella, T. gondii, and P. falciparum all feature a bimodal distribution of simple repeats that is lacking in S. neurona. Finally, all three of the coccidian genomes analyzed here displayed similar distributions of sequence divergence of DNA elements, albeit with slightly different means (22.5, 28.1, and 27.8% for E. tenella, S. neurona, and S. neurona-like DNA repeats in T. gondii, respectively). This suggests that while DNA elements are no longer active in these genomes, they did remain active for slightly longer within E. tenella. From these analyses, we conclude that the maintenance of large numbers of LINEs and DNA elements in S. neurona (and E. tenella), even though they are inactive, likely plays a functional role, since T. gondii has removed most of these elements from its genome.

The S. neurona apicoplast genome is well conserved with other Apicomplexa. In addition to its nuclear genome, the apicoplast genome of S. neurona SO SN1 was studied by reference mapping to the assembled S. neurona SN3 apicoplast sequence (see Fig. S1 in the supplemental material). Both organellar genome architectures are highly similar to those of Toxoplasma (GenBank accession no. U87145.2) and P. falciparum (22). There are, however, a few key differences. As in Toxoplasma, both Sarcocystis apicoplast sequences are missing open reading frame A (ORFA). However, unlike Toxoplasma, both S. neurona sequences show a loss of rpl36 and a loss of one copy of tRNA-Met (from the tRNA cluster between rps4 and rpl4). S. neurona also has a feature first observed in the Piromyalidida that is not seen in Toxoplasma, namely, a division of the RNA polymerase C2 gene into two distinct genes (23). Both S. neurona apicoplast genome sequences uniquely have the insertion of a fragment of rps4 between ORFG and one copy of the large-subunit tRNA in common (see Fig. S1 in the supplemental material). The insert was verified in S. neurona SN3 via PCR and sequencing across this region (see Fig. S2, S3, and S4 in the supplemental material). The rps4 fragment insertion appears to be very recent because the S. neurona SN3 fragment insert is identical in sequence to the corresponding region in the full-length rps4 gene. Comparison of the S. neurona SO SN1 and SN3 nucleotide sequences to each other reveals a few indels but no single-nucleotide polymorphisms (see Text S1 in the supplemental material). Indels, when present, occur in up to one-third of the reads for the locus. The dominant sequence is identical to that determined for SN3. Each S. neurona apicoplast genome was sequenced to greater than 200 × coverage.

The S. neurona genome encodes many novel genes and identifies many coccidian-specific innovations. InParanoid predictions suggest that S. neurona has more orthologs in common with...
Repeat incidence and diversity in selected alveolate genomes. (A) Incidence of repeats, as defined by RepeatModeler (20), in a selected group of alveolate genomes. While *E. tenella* is rich in LTR elements, *S. neurona* is rich in DNA elements. Neither type of element is abundant in other alveolates. (B) Diversity of different repeat families within coccidian genomes. Bar graphs indicate the relative abundance of each repeat class as a function of Kimura divergence from the consensus repeat sequence. Note that DNA elements were not initially detected in the *T. gondii* genome; however, subsequent searches revealed the presence of DNA elements predicted from the *S. neurona* genome (indicated in the inset bar chart of *S. neurona* repeats).
T. gondii than with E. tenella, supporting a closer evolutionary relationship (3,169 versus 1,759 groups of orthologs, respectively) (Fig. 3A). Consistent with previous gene studies, we identified 1,285 (18%) S. neurona genes with no detectable homology (BLAST score, <50) to any known gene, suggesting either a high degree of gene innovation or significant sequence divergence from remote homologs. Among the conserved genes, 715 (10%) were conserved (possessing orthologs) in both Cryptosporidium parvum and either Plasmodium or Theileria annulata, identifying a large collection of proteins that could be amenable to broad-spectrum drug development. These include members of a variety of ATPase genes, heat shock proteins, DEAD/DEAH helicases, proteins with EF-hand domains, and protein kinases. In addition, we identified 1,285 (18%) genes with homology only within the family Sarcocystidae, representing potential drug targets against tissue cyst-forming coccidia. A majority (55%) of these are annotated as “hypothetical proteins” in the ToxoDB resource (24). Of the proteins that are annotated, AP2 domain transcription factors, rhoptry kinase (ROPK) and neck proteins, and zinc fingers, as well as proteins with RNA recognition motifs, are prevalent.

The S. neurona attachment and invasion machinery is broadly conserved with T. gondii. The process of host cell invasion by apicomplexan parasites is a rapid and complex process that relies on a coordinated cascade of interactions between the invading parasite and the host cell. To orchestrate these processes, apicomplexans have evolved families of invasion proteins that are broadly conserved but nevertheless exhibit unique lineage-specific innovations (25). To identify S. neurona gene models involved in invasion relative to T. gondii, we constructed an invasion protein coexpression network (Fig. 3B) in which pairs of T. gondii proteins are linked if they exhibit significant coexpression (Pearson correlation coefficient, >0.8), as has been done for other organisms (26–28). This network provides a scaffold onto which conservation and expression data from S. neurona are mapped to yield insights into evolutionary and functional relationships. Consistent with previous studies, we found that conserved proteins (those that have an ortholog in common with S. neurona) tend to have more correlated expression (high Pearson correlation coefficients) and more connections (high node degree) and are better connected within the network (shorter average path lengths and higher betweenness) than their nonconserved counterparts (Fig. 3C). These findings highlight the potential importance of conserved proteins to the function of the invasion machinery. Within our T. gondii invasion network, we...
identified two main clusters of highly correlated genes associated with key invasion events. The first involves proteins associated with the micronemes (MIC proteins) which strengthen host cell attachment and play a major role in the formation of the moving junction that forms a specific interface, facilitating invasion. The second involves proteins associated with the rhoptries (RON and ROP proteins), an organelle that is absent from the merozoite stage of all Sarcocystis species, including S. neurona (29).

The genome analyses identified nine previously reported S. neurona orthologs of T. gondii MICs (MIC7, MIC8, MIC10, MIC12, MIC13, MIC14, MIC15, MIC16, and M2AP) (30). We also identified potential homologs of MIC2, MIC4, and MIC9 that had not been annotated through the gene model prediction pipeline. MIC4 has previously been shown to form part of a hetero-complex with MIC1 and MIC6 (31). The absence of the latter two homologs from S. neurona suggests that MIC4 likely mediates the more important functional role. MIC7, MIC8, MIC9, and MIC12 are relatively unique in T. gondii with the possession of epidermal growth factor-like domains, suggesting a potential role in ligand binding. MIC10, together with MIC11 (absent from S. neurona) is thought to be involved in the organization of organelar contents. Also secreted by the microneme is apical membrane antigen 1 (AMA1), which functions to link the inner membrane complex (IMC) to the host cell via interactions with RON proteins that together make up the moving junction (6, 32). Our searches revealed two loci, separated by approximately 80 kb on the S. neurona assembly’s largest scaffold, homologous to T. gondii AMA1 protein TgME49_315730. Interestingly, the reading frames of the two S. neurona paralogs (SnAMA1a and SnAMA1b) occur in opposite directions, suggesting an inverted duplication. Supporting this, two inverted repeats >100 bp in length and with >70% identity were identified ~20,000 bp apart, separating the two paralogs. While the region upstream of the paralogs appears repeat rich, containing simple repeats, as well as LINEs and DNA elements, the region downstream of the second paralog is characteristically repeat poor, with no repetitive sequences in ~14,000 bp of sequence. T. gondii possesses additional paralogs of the AMA1 protein, including TgME49_300130. Again, S. neurona appears to possess these two additional AMA1 paralogs (SnAMA1c and SnAMA1d), but in this case, they are present on two different scaffolds. AMA1 has been shown to interact remarkably strongly with RON2, RON4, and RON5 (32).

In general, RONs were well conserved in S. neurona and T. gondii, with RON2, RON3, RON5, and RON8 orthologs displaying significant sequence similarity across their entire length. Three paralogs of T. gondii RON3 (TgRON3) were identified on a single scaffold, suggesting a tandem duplication, two of which appear to be expressed as predicted by the RNA-Seq data. Putative S. neurona orthologs of RON4 and RON6 were identified through manual inspection of sequence alignments. A pattern of conservation and divergence was observed for a putative ortholog of TgRON9. In T. gondii, RON9 and RON10 form a stable complex distinct from the AMA1-RON2/4/5/8 complex, with disruption of either gene leading to the retention of its partner in the endoplasmic reticulum, followed by degradation. This complex does not play a role in T. gondii invasion and virulence but, because of its conservation with C. parvum, has been linked to interactions involving epithelial cells (33). While an S. neurona protein could be aligned over ~25% of the TgRON9 sequence, it was found to lack a single copy of the 22 copies of the PAEENAEFPKQAEEQANASQSET motif associated with the T. gondii protein. No homologs to RON1 or RON10 could be identified.

Another critical organelle required for host invasion is the IMC, which additionally confers stability and shape on the cell and is thought to mediate critical roles in cytokinesis and host cell egress (34). We identified 20 putative S. neurona IMC orthologs, with additional evidence of a further six (see Table S2A in the supplemental material). Only TgGAP70, TgAlv6, and TgAlv7 appear to lack homologs.

**Molecular modeling reveals that SnAMA1a is capable of intimately coordinating SnRON2.** To examine if S. neurona AMA1 homologs can bind S. neurona RON2 homologs, we generated structural models of SnAMA1a and SnAMA1b, which show the highest sequence identity (49 and 44%, respectively) with TgAMA1. Both models possess a PAN-like domain architecture for DI and DII (SnAMA1a, Fig. 4A) consistent with homologs from other apicomplexans (35, 36). A key feature of DII is an extended loop that packs into the groove of DI and regulates RON2 binding in related AMA1 proteins (37). In the SnAMA1a model, a cysteine pair localized within the DII loop is a novel feature of AMA1s and may serve as a hinge to regulate loop displacement and, consequently, RON2 binding (Fig. 4A). Furthermore, the SnAMA1a DII loop appears to be loosely anchored within the DI groove via a Val-Val pair surrounding a central Leu (Fig. 4A). This is in contrast to TgAMA1 (Fig. 4C), where a Trp-Trp pair surround a central Tyr, and the SnAMA1b model (Fig. 4B), where a Trp-Leu pair surround a central Tyr (Fig. 4B). These models suggest that AMA1 paralogs in S. neurona employ divergent strategies that control DII loop dynamics and govern access to the ligand-binding groove. Focusing on the interaction with RON2, removal of the apical segment of the DII loop from the model of SnAMA1a (mimicking the mature binding surface) led to a pronounced groove similar to the RON2 binding surface observed in TgAMA1 (Fig. 4D). Indeed, an energy-minimized docked model revealed that SnRON2 domain 3 was accommodated in a U-shaped conformation (SnRON2D3; Fig. 4E) with an overall topology conserved with respect to the TgAMA1 structure with a synthetic TgRON2D3 peptide (Fig. 4D) (37). Key features of the TgAMA1–TgRON2D3 interface appear to be conserved at the SnAMA1–SnRON2D3 interface, including a RON2 proline residue that occupies an AMA1 pocket exposed by displacement of the DII loop (Fig. 4D and E, yellow arrow) and a reliance on hydrophobic interactions to engage AMA1.

Overall, modeling of apo SnAMA1a and SnAMA1b, in combination with the complex of SnAMA1a with SnRON2D3, supports the hypothesis that these two proteins can form an intact binary complex, as observed in related apicomplexan homologs (37, 38). Of note, both SnAMA1a and SnAMA1b exhibited relatively low levels of expression in the merozoite stage sampled (10.4 and 6.7 fragments per kilobase of exon model per million mapped reads [FPKM], respectively) compared to SnAMA1c and SnAMA1d (63.1 and 83.9 FPKM, respectively), perhaps reflecting a stage-specific role for each AMA1–RON2 pairing.

**Proteins involved in host regulation in T. gondii are not well conserved in S. neurona.** In addition to RONs, rhoptries also secrete a battery of ROP proteins, the products of a group of genes displaying high levels of correlated expression (Fig. 3B). ROP proteins are secreted into the host cytosol to interact with host cell targets, manipulating pathways that protect the intracellular parasite against clearance. To identify putative S. neurona ROP ho-
mologs, we used previously published hidden Markov models (HMMs) (39). In addition to the eight SnROPks reported previously (39), our phylogenetic analysis identified seven new ROPK orthologs, including: ROP20, ROP26, ROP33, ROP34, and ROP45, as well as two SnROPks that appear unique to S. neurona (Fig. 5A). RNA-Seq data support the expression of six of these (ROP14, ROP21, ROP27, ROP30, ROP35, and ROP37) during the merozoite stage, despite this stage’s lack of rhotropies and the ability of schizonts to develop in host cell cytoplasm in the absence of a PV (3).

Overall, S. neurona contains a smaller complement of ROPks (n = 15) than E. tenella (n = 27) and a considerably smaller set than T. gondii (n = 55) and N. caninum (n = 44), both of which feature distinct lineage-specific expansions. However, despite its lower number of ROPks, S. neurona was found to have more ROPks in common with T. gondii and N. caninum than with E. tenella; only two of the ROPks in the three tissue cyst-forming coccidia are conserved with E. tenella (ROP21/27 and ROP35).

Importantly, we did not find S. neurona homologs corresponding to T. gondii ROPk proteins implicated in murine virulence (ROP5 and ROP18), modulation of STAT3 and STAT6 signaling (ROP16), or mitogen-activated protein (MAP) kinase signaling (ROP38), which suggests that S. neurona’s success and pathogenesis are not dependent on the inactivation of these host-specific pathways and may explain, in part, why this parasite is not infectious in rodents. No information is available regarding the functional role of S. neurona ROPks. However, six are likely to be active kinases since they retain key “catalytic triad” residues critical for protein kinase function (SnROP12/27, SnROP30, SnROP33, SnROP34, SnROP35). Further, five are likely to be pseudokinases (SnROP20, SnROP22, SnROP26, SnROP36, SnROP37) that have been shown to act as cofactors of the active kinases (e.g., SnROP5 to SnROP18).

Finally, only two dense-granule (GRA) protein homologs of T. gondii, GRA10 and GRA12, were identified. The discovery of the latter is surprising, given that it has not been annotated in the N. caninum genome. In addition, like the ROPks, the majority of the GRA proteins encoded by T. gondii that specifically target host immune signaling pathways to alter parasite pathogenesis are not encoded by S. neurona. These include GRA6, which regulates the activation of the host transcription factor nuclear factor of activated T cells (NFAT4); GRA15, which regulates NF-κB activation (40); GRA24, which promotes nuclear translocation of host cell p38α MAP kinase (41); and the phosphoprotein GRA25, which alters CXCL1 and CCL2 levels to regulate immune responses and control parasite replication (42). These data indicate either that a different suite of GRA proteins facilitate Sarcocystis host and niche specialization or that Sarcocystis does not require an expanded repertoire of GRA proteins during merozoite replication since it replicates in the host cytosol and is not contained within a PV, like T. gondii or N. caninum.

S. neurona encodes a distinct set of SRS proteins. The SRS proteins exist as a developmentally regulated superfamily of parasite surface adhesins within the tissue cyst-forming coccidia that promote host cell attachment and modulate host immunity to regulate parasite growth and virulence. In previous work, we identified 109 and 246 SRS proteins in the T. gondii and N. caninum genomes, respectively (4, 7). Applying our previously generated HMMs, we identified a more restricted set of only 23 SRS-encoding genes in the S. neurona genome. Twenty of the 23 SRS-encoding genes were distributed across 11 of the major scaffolds, but unlike the SRS-encoding genes in T. gondii and N. caninum, only one genomic locus (SnSRS7 on scaffold 4) existed as a tandem array of duplicated paralogs. The 23 SRS-encoding genes were made up of 75 putative SRS domains (Fig. 5B). Of note, 63 (84%) of these 75 domains were associated with family 2 (fam2) domains, including SRS7A, which contained 26 fam2 domains. In general, each SRS protein possessed either one or two SRS domains, with individual domains classifiable into one of the eight previously defined families, although no fam5 domains were identified. The 26-fam2-domain SRS7A protein genomic locus also contained several gaps bordered by nucleic acids with which a high number of reads could be aligned. This might indicate repetitive elements that could promote domain expansion within this locus through ectopic recombination. Interestingly, the SRS7A protein fam2 domains possessed the highest sequence similarity to the 13 fam2 domains encoded by TgSRS44, a protein previously implicated as an integral structural constituent of the T. gondii cyst wall (43). TgSRS44 also possesses a mucin domain, which has been shown to be highly glycosylated and is thought to protect the cyst from immune recognition and/or dehydration. However, we did not identify any mucin domains in our S. neurona homolog. Only four SRS proteins possessed either fam7 or fam8 domains (one and three copies, respectively), in contrast to T. gondii and N. caninum, where the majority of SRS proteins possess one or the other of these two fam domains. Previous work suggested that the relative expansion of fam7 and fam8 domains in T. gondii and N. caninum is linked to their role in host specificity (7). Other noteworthy features include unique combinations of a fam1 domain with a fam8 domain (SnSRS1), and a fam3 domain with a fam6 domain (SnSRS16), which likely promote specific cell recognition events for S. neurona.

RNA-Seq data identified at least seven SRS proteins expressed in merozoites, which was confirmed by TaqMan reverse transcription-PCR (Fig. 5C). The three most abundantly expressed SnSRS proteins were SnSRS12 (SnSAG3), SnSRS8 (SnSAG2), and SnSRS4 (SnSAG4), as has been observed previously (44). Importantly, the SO SN1 strain did not express SnSRS10 (SnSAG1). SnSRS10 is a highly immunogenic protein and is the major surface antigen expressed on the SN3 strain (45), which explains the high number of SN3-derived expressed se-

FIG 4 SnAMA1a and SnAMA1b accessorize the canonical AMA1 DI and DII domains with unique features but maintain an apical surface capable of coordinating SnRON2D3. (A) Secondary-structure (left) and surface representations of SnAMA1a DI (purple) and DII (orange); five conserved disulfides and two extra cysteines in the DII loop are highlighted as ball-and-stick structures. Two cysteines predicted to form a disulfide at the DII loop hinge are shown as a ball-and-stick structure (black arrow). Residues anchoring the DII loop are labeled and surround a central Leu residue colored yellow. (B) Surface representations of SnAMA1b colored and labeled as for SnAMA1a. (C) Surface representation of TgAMA1 (Protein Data Bank [PDB] accession no. 2x2z); DI, light grey; DII, dark grey. (D) Complementary views of the TgAMA1-TgRON2sp costructure (PDB accession no. 2y8t) with TgAMA1 colored light grey (DI) or dark grey (DII) and TgRON2sp colored cyan. (E) Complementary views of the SnAMA1a-SnRON2D3 costructure model, with SnAMA1 colored as in panel A and SnRON2D3 in green. Residues making up the RON2 cystine loop tip are shown as ball-and-stick structures to highlight shape complementarity.
Coccidian-specific protein families implicated in virulence and host range determination. (A) Maximum-likelihood-based phylogenetic tree of the ROPK family. Values indicate the bootstrap support (of 1,000 replicates). S. neurona members are red. T. gondii members are dark blue. N. caninum members are cyan. T. gondii and N. caninum clades are blue. E. tenella members and clades are yellow. (B) Summary of the 23 SRS family members identified in the S. neurona genome. Relative expression in the S. neurona merozoite stage are provided as FPKM values, and domain architectures are indicated. #ESTs, number of expressed sequence tags. (C) The expression of the SnSRS-encoding genes was assessed by TaqMan qPCR. Genes were sorted in descending order by their expression levels.

FIG 5 Coccidian-specific protein families implicated in virulence and host range determination. (A) Maximum-likelihood-based phylogenetic tree of the ROPK family. Values indicate the bootstrap support (of 1,000 replicates). S. neurona members are red. T. gondii members are dark blue. N. caninum members are cyan. T. gondii and N. caninum clades are blue. E. tenella members and clades are yellow. (B) Summary of the 23 SRS family members identified in the S. neurona genome. Relative expression in the S. neurona merozoite stage are provided as FPKM values, and domain architectures are indicated. #ESTs, number of expressed sequence tags. (C) The expression of the SnSRS-encoding genes was assessed by TaqMan qPCR. Genes were sorted in descending order by their expression levels.
sequence tags that mapped to SnSRS10, which was transcriptionally silent in this study (Fig. 5).

Reconstruction and analysis of *S. neurona* metabolism reveal the potential to exploit alternative sources of energy. *S. neurona* has 372 metabolic enzymes (unique enzyme classification [EC] numbers, excluding those involved in nonmetabolic reactions) in common with *T. gondii* but is missing 42 enzymes and has an additional 13 enzymes that are expressed by RNA-Seq in the merozoite stage (Fig. 6A; see Text S1 in the supplemental material). Our analyses predict putative *T. gondii* orthologs for 12 of these enzymes, including the fatty acid elongation genes very-long-chain 3-oxoacyl coenzyme A synthase (EC 2.3.1.199; TGME49_205350) and very-long-chain (3R)-3-hydroxyacyl acyl carrier protein dehydratase (EC 4.2.1.134; TGME49_311290). Only threonine ammonia-lyase (EC 4.3.1.19) is unique and adds functionality to *S. neurona*.

We incorporated these differences into our previously published metabolic reconstruction of *T. gondii* named iCS382 (8) and performed flux balance analyses of both iCS382 and the modified *S. neurona* reconstruction. Scaling the iCS382 model to produce a doubling time of 11.8 h with glucose as the sole energy source (see Text S1 in the supplemental material), we show that *S. neurona* has a slightly longer doubling time of 13.8 h. Single-reaction knockouts identified 22 reactions whose deletion resulted in a significantly greater impact on *S. neurona* than on *T. gondii* (>20% maximal growth rate difference) (Fig. 6B; see Table S2 in the supplemental material). Critical reactions include members of the pentose phosphate and glycolysis pathways, the tricarboxylic acid (TCA) cycle, and two members of the pyrimidines biosynthetic pathway, nucleoside-diphosphate kinase (EC 2.7.4.6) and cytidylate kinase (EC 2.7.4.14). Conversely, we identified only one single reaction, catalyzed by pyruvate dehydrogenase, whose deletion had a significantly greater impact on *T. gondii* than on *S. neurona* (>20% maximal growth rate difference).

The *S. neurona* annotation effort predicted a gene for alpha-glucosidase (EC 3.2.1.20) (see Text S1 and Fig. S4 in the supplemental material). Since conversion of sucrose to fructose and glucose by alpha-glucosidase would add functionality to the metabolic reconstruction, we tested *in silico* for its potential impact on growth. *S. neurona* was predicted to grow faster in the presence of sucrose and the absence of glucose than in the presence of glucose and the absence of sucrose (doubling time of 11.4 h versus 13.8 h). This is due, in part, to an increase in the concentration of fructose-6-phosphate caused by the action of hexokinase (EC 2.7.1.1, Fig. 6C). Consequently, under conditions of sucrose uptake, knockout of enzymes involved in glycolysis has a greater impact on the growth rate than conditions of glucose uptake (see Table S2 in the supplemental material). When we examined the impact of combining access to different carbohydrates, our simulations suggested that *S. neurona* has the capacity to significantly enhance its growth by utilizing fructose, with an even greater effect when sucrose is used as an additional energy source (Fig. 6D). For example, while fructose supplementation alters parasite growth to 120%, supplementation with sucrose extends parasite growth to 180% of its original rate. Interestingly, glucose-6-phosphate isomerase, the enzyme responsible for the conversion of glucose-6-phosphate to fructose-6-phosphate, operates in the reverse direction under glucose or sucrose uptake conditions. When only sucrose is available, more glucose-6-phosphate is produced from the conversion of fructose-6-phosphate, which is predicted to feed into other pathways (e.g., the pentose phosphate pathway), resulting in the elevated production of NADPH and an increased growth rate. Importantly, glycolysis is utilized more when sucrose is available, so there is less reliance on the TCA cycle. Furthermore, the breakdown of sucrose makes fructose available for the synthesis of other key metabolites (e.g., branched-chain amino acids), decreasing the parasite’s dependency on the TCA cycle for their production. Hence, the deletion of individual TCA cycle reactions has a greater impact on the growth rate in the presence of glucose than in the presence of sucrose (Fig. 6C).

**DISCUSSION**

Coccidian parasites represent a major clade within the phylum *Apicomplexa*, and the genomes of three species, *E. tenella*, *T. gondii*, and *N. caninum*, have already been sequenced (7, 21). *S. neurona* is the first genome in the genus *Sarcocystis* to be sequenced. The 127-Mbp genome is more than twice the size of other sequenced coccidian genomes, largely because of a high proportion of repetitive LINEs and DNA elements. The organization of the *S. neurona* genome into 116 genomic scaffolds produces the first molecular karyotype, or physical linkage map, which should greatly facilitate future genetic and comparative genomic studies of this important genus. *Sarcocystis* chromosomes do not condense, nor have they been resolved by pulse-field gel electrophoresis. Our comparative genomic, transcriptomic, and metabolic flux data analyses show that the invasion machinery is largely conserved among the coccidia but that the tissue cyst-forming coccidia have evolved families of dense-granule (GRA), ROPK, and surface-associated SRS adhesins that promote their ability to persist chronically in cyst-like structures or disrupt the induction of sterilizing immunity, representing novel molecular strategies that facilitate their transition from largely enteric pathogens within a single host (*Eimeria*) to heteroxenous pathogens that cycle between a definitive host and an intermediate host(s) (*Sarcocystis*).

Genome comparisons reveal that *S. neurona* has more orthologs in common with *T. gondii* than with *E. tenella* (3,169 versus 1,759 orthologs, respectively), supporting the notion that the *Eimeria* lineage is more divergent. However, *S. neurona* is also quite distinct from *T. gondii*; it possesses only limited genomic synteny, restricted to only dozens of genes, and additionally encodes 1,285 (18%) genes with no detectable homology to any other species. As in *E. tenella*, LINEs and DNA elements are absent in *S. neurona*, but the DNA elements are significantly expanded in *S. neurona*, partially accounting for its increased genome size. The presence of the LINEs and DNA elements, however, is not associated with gene model misannotation, since LINEs are as frequently associated with *T. gondii* orthologs (13.3%) as they are with unique genes (11.6%), indicating that they may drive genome innovations within *S. neurona* (46). We did not find any examples of the coronavirus-like long terminal repeat (LTR) element previously associated with the *E. tenella* genome (21), strengthening the suggestion that this element was acquired by horizontal gene transfer within that lineage.

The *Sarcocystis* invasion machinery was largely conserved within the coccidia, and the construction of the *S. neurona* interaction network based on gene expression data identified two main clusters of conservation, one composed largely of MIC, AMA1, and RON proteins required for the mechanics of cell attachment and invasion and another composed of a limited set of ROP and GRA proteins thought to alter host immune effector function.
FIG 6 Metabolic reconstruction and analysis of S. neurona based on iCS382. (A) Overlap in enzyme predictions for genes from S. neurona, T. gondii, and P. falciparum. (B) Species-specific differences in growth rates of single-reaction knockouts. Only reactions that show a growth rate difference of 20% between T. gondii and S. neurona are shown. (C) Impact of deletion of reactions involved in glycolysis and the TCA cycle on S. neurona growth under conditions of exclusive glucose or sucrose uptake. D-glc-6-P, D-glucose-6-phosphate; G6PI, glucose-6-phosphate isomerase; D-frc-6-P, D-fructose-6-phosphate; DF6P1P, (Continued)
However, the complement of the latter ROP and GRA proteins is greatly reduced compared to that of other tissue cyst-forming coccidia such as *Toxoplasma* or *Neospora*. While mouse models have shown ROP5 and ROP18, which are absent from *S. neurona*, to impact virulence in *Toxoplasma*, the lack of a suitable such model, i.e., immunocompetent mice, for *S. neurona* means that little is known about its strain virulence determinants. Additionally, all strains induce fatal encephalitis in immunodeficient mice, irrespective of the dose. Only two ROPs were conserved with *E. tenella*, implying specialization in the ROPK machinery required for the different life cycles. Hence, the reduced complement of ROPKs within the *S. neurona* genome likely underscores the important role the expanded repertoire of ROPKs plays in promoting *Toxoplasma* and *Neospora* host and niche adaptation among the susceptible hosts in which these parasites establish transmissible infections. Likewise, the distinctive set of ROPKs previously reported for *E. tenella* and thought to map to the sporozoite rhoptry (47) might suggest a specialized role for these proteins during the initial establishment of infection.

Consistent with a transition from a strictly enteric coccidian pathogen to a tissue-invasive one capable of establishing long-term, chronic infection by encystment within host cells, *S. neurona* expresses a distinct surface antigen coat of SRS proteins that promote parasite recognition, attachment, and long-term encystment within host cells to promote transmissibility of infection. In comparison to *T. gondii* and *N. caninum*, however, the *S. neurona* SRS protein repertoire is surprisingly small and less divergent (25); there is a dramatic reduction in the number of SRS proteins composed of fam7 and fam8 domains, with the vast majority of the 23 SnSRS-encoding genes composed of fam2 domains. Previous studies of *T. gondii* suggest that proteins composed of the former domains modulate host immune responses and mediate critical roles in parasite virulence. Our data suggest that with only a single copy of a fam7- and fam8-containing SRS protein, *S. neurona* has evolved other mechanisms for control of immune activation and/or that such control is not required for the successful transmission of this highly prevalent protozoan pathogen. The latter point is consistent with observed differences between the *S. neurona* and *Toxoplasma/Neospora* life cycles. *Sarcocystis* spp., once encysted, undergo a terminal commitment to their gamont stage, requiring access to their definitive host to complete their life cycle. In contrast, both *Toxoplasma* and *Neospora* are capable of recrudescing their infection after encystation, and expansion of fam7 and fam8 domain SRS proteins capable of altering host protective immunity may function to increase the cyst burden or alter intermediate-host behavior, promoting transmission of the parasite to the definitive host to complete its life cycle. Alternatively, *Sarcocystis* spp. are exclusively restricted to sexual development within the intestine of the definitive host, whereas *Toxoplasma* infection of its felid definitive host results in both sexual development and asexual expansion of infection, so the expanded repertoire of fam7 and fam8 domain SRS proteins may promote dissemination of infection to a wide range of tissue and cell types and vaccinate the definitive host against reinfection.

The sheer dominance of the fam2 domain proteins among the limited repertoire of SnSRS proteins suggests that they play a critical functional role in the life cycle. A recent study (43) found that TgSRS44 (CST1), a *T. gondii* SRS protein with 13 tandemly repeated fam2 domains, is an important structural constituent of the cyst wall, suggesting that the emergence of fam2 SnSRS domain-containing proteins in the common ancestor of *S. neurona* and *T. gondii* likely provided the parasite with the ability to form cysts, thereby extending its host range and promoting the transition to a heteroxenous (two-host) life cycle. Strains of *S. neurona* are known to show important differences in the immunodominant SnSRS-encoding genes that they possess. SnSRS10 (SnSAG1) is encoded by an immunodominant gene present and expressed abundantly in some *S. neurona* isolates but absent from others (45). While the type II SO SN1 strain sequenced encodes SnSRS10, it does not express it during merozoite growth (Fig. 5), whereas SN3, another type II isolate, highly expresses this protein. While the mechanism of gene regulation within the SnSRS family has yet to be elucidated, it may influence the host range, the capacity to promote coinfection, and/or pathogenicity among the broad intermediate-host range of *S. neurona*, much the same way differential expression of TgSRS2 alters the parasite load and the pathology of *T. gondii* infection in mice (4). Importantly, a high prevalence of coinfection with different genetic types of *S. neurona* within intermediate hosts would promote outcrossing during sexual reproduction. Outcrossing in *Toxoplasma* has previously been shown to produce progeny possessing altered biological potentials, including virulence and a capacity to cause outbreaks (48), which has recently also been established for *S. neurona* (15).

Finally, regulation of energy production has likewise evolved as a strategy for parasites to extend their host range, by tuning growth in relation to the host burden or carrying capacity (8). We found only a limited number of differences between the enzyme complements of *T. gondii* and *S. neurona*. Notably, *S. neurona* possesses 13 enzymes not present in *T. gondii* and a homolog of an alpha-glucosidase (EC 3.2.1.20) that preferentially gives sucrose and that sucrose supplementation can increase parasite growth. Hence, our flux balance analysis showed that *S. neurona* is less reliant on the TCA cycle when it is grown in the presence of sucrose and that sucrose supplementation can increase parasite growth to 180% of its original rate, a capability that may be important for allowing the parasite to exploit new host niches. These findings serve to highlight subtle differences in pathway utilization that the two parasites may have adopted to optimize their distinct life cycle strategies.

Together, our data support a model in which, following the split with the *Eimeria* lineage, the ancestor of *Sarcocystis* and *Toxoplasma* gained the ability to invade intermediate hosts and form tissue cysts. This transition required the evolution of SRS family proteins as structural constituents of the cyst wall, as well as immune evasion molecules protecting the parasite from sterilizing immunity. Subsequently, while the *Sarcocystis* lineage abandoned the use of the PV during its schizont stage in the intermediate host, committing the parasite to its sexual cycle after encystation, the *Toxoplasma* lineage...
maintained the use of a PV during intermediate-host infection. The use of the PV could conceivably shield the parasite from the host developing an effector memory CD8 T cell response that is naturally induced by the presence of parasite antigens in the cytosol of infected host cells. This, in turn, allows *Toxoplasma* to recrudescence postcystation and, aided by an expanded repertoire of ROPK, GRA, and SRS proteins, provides further opportunities to increase the cyst burden and extend its host range. In addition to addressing questions of host range and specificity, we expect that the availability of this resource will help drive the development of novel therapeutics that are urgently required for these devastating pathogens. Further, reference genome mapping will facilitate genus-wide and population studies that focus on questions of host specialization and virulence mechanisms. The latter, for example, might be expected to inform on the fate of fatal infections in marine mammals to resolve at the genome level the genetic basis of the emergence of these disease-producing strains. Key to these studies will be the generation of robust expression data sets that allow the identification of critical proteins associated with distinct stages of the parasite’s life cycle.

**MATERIALS AND METHODS**

Culturing of parasites, extraction of DNA/RNA, and sequencing. *S. neurona* strain SO SN1 was isolated from a southern sea otter (19) and obtained from Patricia Conrad, University of California, Davis, CA. *S. neurona* parasites were maintained in MA-104 cells as described previously (49). Genomic DNA was extracted from frozen pellets of *S. neurona* SO SN1 by proteinase K digestion and subsequent phenol-chloroform extraction. Five libraries were prepared: two Roche 454 Shotgun GS-Titanium libraries prepared in accordance with the Rapid Library Preparation Method Manual (Roche), a Roche 454 8-kb paired-end GS-Titanium Library prepared in accordance with the Paired-End Library Preparation Method Manual with the modification of setting up four circularization reactions to increase the final library yield, an Illumina 2- to 3-kb mate pair library synthesized with the TrueSeq DNA sample prep kit (Illumina) and run on an Illumina GA IIx, and a Nextera 8- to 15-kb mate pair library prepared in accordance with the manufacturer’s recommendations and run on an Illumina HiSeq 2000. These sequencing efforts generated 7,020,033 shotgun reads, 5,919,255 shotgun reads, 1,100,788 paired-end reads, 128,614,194 mate pair reads, and 136,301,151 mate pair reads, respectively. *S. neurona* SO SN1 RNA was isolated from merozoites with the RNeasy mini kit (Qiagen), snap-frozen, and stored at −80°C. A single TrueSeq v2 RNA library (mRNA enriched) was prepared for Illumina sequencing by the standard Illumina protocol and used to generate 59,622,019 reads. For further details of genome assembly and annotation, as well as bioinformatics and experimental analyses, see Text S1 in the supplemental material.

**Nucleotide sequence accession number.** Further information on the *S. neurona* genome project, including sequence files, is available through the bioproject repository at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/bioproject/252030) using the accession number SRP052925.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.orglookup/suppl/doi:10.1128/mBio.02445-14/-/DCSupplemental. Text S1, PDF file, 0.3 MB. Figure S1, PDF file, 0.6 MB. Figure S2, PDF file, 0.3 MB. Figure S3, PDF file, 0.3 MB. Figure S4, PDF file, 0.2 MB. Figure S5, PDF file, 1.3 MB. Table S1, XLS file, 1.8 MB. Table S2, PDF file, 0.3 MB. Table S3, XLSX file, 0.04 MB. Table S4, PDF file, 0.2 MB.

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