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Ferritin 1 silencing effect in *Rhipicephalus sanguineus* sensu lato (Acari: Ixodidae) during experimental infection with *Ehrlichia canis*

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ABSTRACT

Rhipicephalus sanguineus sensu lato (s.l.) is a very common ectoparasite of domestic dogs able to transmit several pathogens of human and veterinary importance. Tick infestations and tick-borne diseases (TBDs) remain a serious and persistent problem, due to the lack of efficient control measures. It is therefore vital that novel approaches to control are pursued. Whilst vaccination is recognised as a potential control method to reduce tick infestation, no anti-*R. sanguineus* vaccine is available. Ticks depend on their blood meals to obtain nutrients and to achieve sexual maturity, which exposes them to vast amounts of iron. Although an essential molecule for several biological processes, its excess can lead to oxidative stress. Iron homeostasis is achieved with the help of iron-binding proteins called ferritins, among others, present in several tick tissues and developmental stages. These evolutionarily conserved proteins regulate iron homeostasis by storing and releasing iron in a controlled manner. In this study the *R. sanguineus ferritin 1* gene was silenced through RNA interference (RNAi) in adult females exposed to an experimental infection with *Ehrlichia canis.* The purpose of this study was to assess the role of this protein in tick feeding, ovary development, oogenesis, and pathogen acquisition. Our data has shown that silencing *ferritin 1* alters tick competence to normally engorge and causes morphologic and histochemical changes in the ovaries (OV) and oocytes. Furthermore, our data revealed that no *E. canis* DNA was found in either experimental group. Determining the function of molecules that act in key biological processes, such as blood digestion or reproduction, and that could be considered potential tick antigens will contribute towards the improvement of current control measures against these ectoparasites and the pathogens they vector.

Introduction

Rhipicephalus sanguineus s.l. is a very common ectoparasite of domestic dogs (Dantas-Torres, 2008) and the most widespread tick in the world, being particularly prevalent in tropical and subtropical regions (Aguirre et al., 2004; Dantas-Torres, 2010; Estrada-Pena and Jongejan, 1999; Hua et al., 2000; Keefe et al., 1982; Woldehiwet and Ristic, 1993) Considered a monotropic tick, all stages of *R. sanguineus* s.l. prefer dogs as primary hosts in both urban and rural areas, even though they could feed on other wild and domestic animals (Dantas-Torres, 2010). Immature stages can occasionally be found on rodents and other small mammals such as rabbits, cats, pigeons, wild canids and humans; whereas adults prefer larger mammals (Bouloy et al., 1994; Breitschwerdt et al., 2002; Maxwell, 2006). *R. sanguineus* s.l. is known to act as the vector of several infectious pathogens, including *E. canis*, the

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etiological agent of canine monocytic ehrlichiosis (CME) (Ewing, 1969; Groves et al., 1975; Harvey et al., 1979), *Babesia canis* (Regendanz and Muniz, 1936) and *B. gibsoni* (Sen, 1933), the agents of canine babesiosis, and of *Coxiella burneti*, the agent of Q fever (Mantovani and Benazzi, 1953), *Hepatozoon canis*, the agent of canine hepatozoonosis (Nordgren and Craig, 1984), *Rickettsia conorii*, responsible for Mediterranean spotted fever (Brumpt, 1932) and *R. rickettsii*, the agent of Rocky Mountain spotted fever (Parker et al., 1933). Recently, it has been suspected that *R. sanguineus* s.l. could also be involved in the transmission of *Leishmania infantum*, the etiological agent of visceral leishmaniasis (Coutinho et al., 2005).

Current methods of controlling ticks and TBDs rely heavily on chemical acaricides; however, their continued use is unsustainable not only due to environmental contamination but also due to the emergence of resistance caused by their intense and indiscriminate use, that most likely has selected tick populations with mutations in the genes that encode detoxifying enzymes; or that suffered genetic drift (Ghosh et al., 2007; Rodriguez-Vivas et al., 2011; Rosario-Cruz et al., 2009). Alternative cost-effective and environmentally friendly control measures such as anti-tick vaccines are urgently needed. The use of vaccines to reduce tick infestations is a well-established concept. The lack of highly efficacious antigens, however, remains a major limitation to developing vaccines that induce protective immune responses whilst preventing pathogen transmission (de la Fuente and Kocan, 2006). To date, no specific anti- *R. sanguineus* vaccine has been commercially developed.

Ticks are obligatory blood-sucking arthropods that depend completely on their hosts to obtain nutrients and, in the case of adult females, to achieve sexual maturity (Galay et al., 2013). During each blood meal, ticks are exposed to iron molecules that are required as biological cofactors for metabolic and transport functions, amongst others. At the same time, the excess of iron derived from the host blood can act as a highly toxic precursor of reactive oxygen species through the catalysis, propagation and production of reactive radicals (Wang and Pantopoulos, 2011). It also seems that ticks lack an efficient system to excrete iron through the faeces (Sonenshine, 1991). To prevent the oxidative damage to proteins, lipids and DNA caused by excess iron, and to establish iron homeostasis during a blood meal, ticks can store and metabolise these ions with the help of iron-binding ferritin proteins. These proteins are ubiquitously present in different tissues and at different developmental stages (Galay et al., 2013). An intracellular ferritin (FER1) that stores iron within cells, and a secretory type ferritin (FER2) that concentrates and secretes it from gut cells to the hemolymph, have been previously described in some hard ticks (Galay et al., 2013; Hajdusek et al., 2009). As antioxidants with different functions, both influence blood feeding, reproduction and survival, reinforcing the idea that these iron-binding proteins are strong candidates for further investigation for developing anti-tick antigens. Two recent studies have explored the ability of ferritins to elicit anti-tick immune responses. In rabbits immunised with two recombinant tick ferritins, one of these (H1FER2) offered some protection (Galay et al., 2014a). A similar study where cattle and rabbits were immunised with recombinant ferritins derived from different tick species, found the ferritin 2 (FER2) protein conferred some protection in both mammals (Hajdusek et al., 2010). These studies demonstrate the characterisation of highly conserved, fundamentally important proteins, such as ferritins, could contribute towards the improvement of current control measures.

In this study, RNAi experiments were conducted in *R. sanguineus* s.l. adult females fed on an *E. canis*-infected dog to evaluate the biological importance of *ferritin 1* in tick feeding, ovary development, oogenesis, and pathogen acquisition.

2. Material and methods

2.1. Ticks and bacteria

The laboratory pathogen-free colony of *R. sanguineus* s.l. ticks (Tropical lineage) (GenBank no. KC018070 and JX997391) (Sanches, 2013; Sanches et al., 2012) was maintained at the Department of Veterinary Pathology, FCAV-UNESP, Jaboticabal, Brazil. Briefly, ticks were kept in a chamber regulated at 27 ± 1 °C, 80% relative humidity and a photoperiod of 12:12 (light: dark), and maintained by feeding on the ears of 5–8 month-old New Zealand white rabbits. For clarification, the tropical lineage of *R. sanguineus,* but not the temperate lineage, has been shown to be a competent vector of *E. canis* (Moraes-Filho et al., 2015).

E. canis Jaboticabal strain (GenBank no. DQ401044) was isolated from a Weimaraner dog blood sample during the acute phase of CME infection, in 1993 (Rosângela Z. Machado; unpublished data). Currently, the strain is maintained in the canine monocyte-macrophage cell line DH82 (Wellman et al., 1988) at the Immunoparasitology Laboratory, UNESP, Jaboticabal, São Paulo, as described elsewhere (Aguiar et al., 2007a,b).

2.2. Experimental animals

A two month-old male German shepherd dog was acquired from a certified breeder andconfirmed to be ehrlichiosis and ectoparasite-free. As an additional measure, 5 mL of blood were taken and tested for the presence of *Neospora caninum* (Mineo et al., 2009), *Toxoplasma gondii* (Domingues et al., 1998), *E. canis* (Andre et al., 2010) and *B. vogeli* (Furuta et al., 2009) by Indirect Fluorescent Antibody Test (IFAT), as previously described. Blood was also tested for the presence of *E. canis* (Murphy et al., 1998) and *B. vogeli* (Jefferies et al., 2007) by PCR. For the template, genomic DNA was extracted from whole blood using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. All of these tests were negative. The animal was also vaccinated with Vanguard Plus 5® (Zoetis, New Jersey, USA) and RecombiteK C6/CV® (Merial, Georgia, USA), with a 3-week interval, and dewormed. Dry food was provided twice a day and water *ad libitum*. For the infection, the dog was inoculated intravenously with a dose of 4.5 mL *E. canis* purified from DH82 cells as described elsewhere (Aguiar et al., 2007a,b). When the experimental infection took place, the dog was 5 months-old. Following inoculation, the infection was monitored by qPCR for the *dsb* gene (Doyle et al., 2005) and Giemsa-stained blood smears throughout the whole experimental period to confirm the presence of *E. canis* in the dog. The qPCR was positive for *dsb* transcripts from day 3 post-inoculation. To detect the presence of morulae in Giemsa-stained capillary blood smears by light microscopy (Olympus CX31, Olympus, Tokyo, Japan), blood was taken from the ear tip, up until parasitaemia was confirmed on day 17 post-inoculation. The experimental animals were treated following the approved guidelines from the Lei Arouca 11.794/08, in Brazil.

2.3. dsRNA synthesis and RNA interference

The *ferritin 1* mRNA sequence (GenBank accession number: EZ406186.1) was obtained from a *R. sanguineus* s.l. salivary gland (SG) transcriptome database (Anatriello et al., 2010). Primers (Ferritin_T7_F/Ferritin_T7_R) were designed against this sequence to include the T7 promoter sequence (Table 1). Total RNA was extracted from *R. sanguineus* SG using TriReagent (Sigma-Aldrich, St. Louis, USA) according to the manufacturer's instructions. cDNA was synthesised from 100 nanograms (ng) of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, California, USA) following the manufacturer's protocol. Using the aforementioned primer set, PCR