Azadirachtin interferes with basal immunity and microbial homeostasis in the *Rhodnius prolixus* midgut

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ABSTRACT

Rhodnius prolixus is an insect vector of two flagellate parasites, *Trypanosoma rangeli* and *Trypanosoma cruzi*, the latter being the causative agent of Chagas disease in Latin America. The *R. prolixus* neuroendocrine system regulates the synthesis of the steroid hormone ecdysone, which is essential for not only development and molting but also insect immunity. Knowledge for how this modulates *R. prolixus* midgut immune responses is essential for understanding interactions between the vector, its parasites and symbiotic microbes. In the present work, we evaluated the effects of ecdysone inhibition on *R. prolixus* humoral immunity and homeostasis with its microbiota, using the triterpenoid natural product, azadirachtin. Our results demonstrated that azadirachtin promoted a fast and lasting inhibitory effect on expression of both *RpRelish*, a nuclear factor kappa B transcription factor (NF-kB) component of the IMD pathway, and several antimicrobial peptide (AMP) genes. On the other hand, *RpDorsal*, encoding the equivalent NF-kB transcription factor in the Toll pathway, and the *defC* AMP gene were upregulated later in azadirachtin treated insects. The treatment also impacted on proliferation of *Serratia marcescens*, an abundant commensal bacterium. The

simultaneous administration of ecdysone and azadirachtin in *R. prolixus* blood meals counteracted the azadirachtin effects on insect molting and also on expression of *RpRelish* and AMPs genes. These results support the direct involvement of ecdysone in regulation of the IMD pathway in the *Rhodnius prolixus* gut.

Keywords: Ecdysone, Antimicrobial peptides, immune signalling pathways, Rhodnius prolixus, Serratia marcescens, microbiota.

INTRODUCTION

The triatomine *Rhodnius prolixus* is one of the main vectors of *Trypanosoma cruzi*, the etiological agent of Chagas disease in the Americas (Coura, 2015). In the American continent, Chagas disease has been highlighted as a public health problem since its control involves educational strategies as well as costly government investment, such as the application of insecticides and the improvement of housing where triatomine vectors cohabit with men (Coura, 2015; Coura and Dias, 2009). It is common knowledge that *T. cruzi* develops exclusively inside the triatomine digestive tract (Dias Fde et al., 2015; Ferreira et al., 2016; Garcia et al., 1999; Gonzalez et al., 1999) where it needs to overcome the activation of innate immune responses, such as antimicrobial peptides (AMPs), lysozymes and the prophenoloxidase cascade while interacting with the intestinal microbiota(Azambuja et al., 2004; Azambuja et al., 2017; Castro et al., 2012; Mello et al., 1996; Vieira et al., 2016).

The synthesis of the steroid hormone ecdysone is crucial for insect development and molting (Kozlova and Thummel, 2000; Riddiford, 1993; Yamanaka et al., 2013). This hormone is produced by prothoracic glands and is posteriorly released into the hemolymph, where it circulates through the body of the insect and binds to its nuclear specific receptor EcR (ecdysone receptor), present in several tissues (Henrich, 2005; Vafopoulou and Steel, 2006; Vafopoulou et al., 2005). Ecdysone pulses trigger a cascade of gene expression, which ultimately induces the physiological alterations related to molting and metamorphosis (Henrich, 2005; Thummel, 1996; Yamanaka et al., 2013). Besides that, ecdysone is known to influence several other aspects of insect physiology, including embryogenesis (Wang et al., 2018), behavioral biology (Ishimoto and Kitamoto, 2011) reproductive and digestive systems (Albuquerque-Cunha et al., 2004; Lenaerts et al., 2019) and innate immunity (Azambuja et al., 1997).

Azadirachtin (AZA) is a triterpenoid compound extracted from the neem tree *Azadirachta indica* (Rembold, 1987). AZA affects the insect neuroendocrine system interfering with the release of the prothoracicotropic hormone (PTTH) from neurosecretory cells, which in turn reduces ecdysone synthesis (Garcia et al., 1987; Garcia et al., 1990). Moreover, AZA can interact directly with the ecdysone receptor (EcR) as an ecdysone antagonist, which in turn impairs the function of the hormone (Oliveira, 2019). AZA added to the blood meal of *Rhodnius prolixus* nymphal stages reduces levels of ecdysteroids in the hemolymph and disrupts the insect molt process (Garcia et al., 1987; Garcia et al., 1991). Also, the treatment of fifth instar nymphs of *R. prolixus* with AZA results in the prevention of the *Trypanosoma cruzi* development (Albuquerque-Cunha et al., 2004; Gonzalez and Garcia, 1992; Gonzalez et al., 1999). All these effects caused by AZA, including blocking of molting and *T. cruzi* adhesion to the midgut lumen surface, can be counteracted by concomitant treatment of *R. prolixus* with azadirachtin plus ecdysone (Garcia and H, 1984; Garcia ES, 1984. ; Gonzalez et al., 1999). Although the inhibitory effects of AZA on the *R. prolixus* neuroendocrine system and *T. cruzi* infection have been partially elucidated (Gonzalez et al., 1999; Nogueira et al., 1997), the role of this drug and the reversion effects of ecdysone on gut microbiota and its homeostasis are not entirely understood.

Besides its role in insect development and reproduction, ecdysone is also associated with the regulation of the immune system in various species (Ahmed et al., 1999; Azambuja et al., 1997; Dimarcq et al., 1997; Han et al., 2020; Han et al., 2017; Ma et al., 2019; Meister and Richards, 1996; Tian et al., 2010). In *Drosophila melanogaster*, ecdysone has a role in the insect's humoral response; both in vivo and in vitro treatments with ecdysone increase levels of expression of antimicrobial peptides (AMPs) (Flatt et al., 2008; Rus et al., 2013; Zhang and Palli, 2009). In *R. prolixus,* inhibition of ecdysone by AZA treatment leads to an inhibition of cellular immune responses, such as hemocyte aggregation counts (nodule formation), thereby increasing insect mortality after a bacterial challenge (Azambuja, 1991; Azambuja et al., 1997). Hemocytes obtained from *R. prolixus* previously treated with AZA exhibited reduced *Saccharomyces cerevisiae* phagocytosis when compared to the hemocytes from control insects (Figueiredo et al., 2006). Ecdysone added to *R. prolixus* blood meals concomitantly with AZA was able to counteract those effects corroborating the role of this hormone as a mediator of the immune response in *R. prolixus* (Azambuja, 1991; Azambuja et al., 1997; Figueiredo et al., 2006).

The humoral immune response is also affected by AZA treatment with a decrease of lysozyme, phenoloxidase, and antibacterial activities in the *R. prolixus* hemolymph (Azambuja, 1991; Figueiredo et al., 2006). However, the effect of ecdysone on the regulation of AMPs in triatomines has not been explored until now. The regulation of antimicrobial peptides (AMPs) synthesis is involved in interactions of the host with Gram-positive and Gram-negative bacteria, *T. cruzi* and *T. rangeli*, as well as in the modulation of intestinal microbiota (Vieira et al., 2015; Vieira et al., 2016; Vieira et al., 2014).

The *R. prolixus* intestinal microbiota includes species of *Serratia*. Indeed, *Serratia* species have been identified as commensals of several different triatomines (Gumiel et al., 2015) in the wild and also in various insectary colonies (da Mota et al., 2012). *Serratia* species have been reported as symbionts in some insects such as aphids (Manzano-Marín et al., 2016), but can be pathogenic to other insects such as mosquitões (Bahia et al., 2014) and flies (Benoit et al., 1990; Lauzon et al., 2003). The commensal *Serratia* species that colonise the midgut of *R. prolixus* express trypanolytic activities towards *T. cruzi* and can thereby modulate infection of the insect by the parasite (Azambuja et al., 2004; da Mota et al 2019). As such, these bactéria play an importante role in maintaining homeostasis of the *R. prolixus* intestinal microbiota.

In general, the expression of AMPs is regulated in response to Toll, IMD, and Jak-Stat signaling pathways (Ferrandon et al., 2007; Salcedo-Porras and Lowenberger, 2019). Pathogen Associated Molecular Patterns (PAMPs) can be detected by pattern recognition receptors (PRRs) in the insect hemocoel or gut, and transcription factors of the NF-κB family are activated and translocated to the nucleus inducing AMP expression (Ferrandon et al., 2007; Huxford and Ghosh, 2009; Mesquita et al., 2015; Silverman and Maniatis, 2001; Vieira et al., 2015; Vieira et al., 2016). Two cascades are involved in AMP expression: Toll and immune deficiency (IMD) pathways (Ferrandon et al., 2007; Salcedo-Porras and Lowenberger, 2019), and some components of these pathways were recently discovered in *R. prolixus* (Mesquita et al., 2015; Nishide et al., 2019; Ribeiro et al., 2014; Ursic-Bedoya et al., 2009). Analyses of the expression of *Rpdorsal*, a canonical NF-κB component of the Toll pathway, and *Relish*, an NF-κB component of IMD cascade, provide evidence of these activation mechanisms (Salcedo-Porras et al., 2019; Vieira et al., 2018). The importance of these signaling pathways in the establishment of the *T. cruzi* infection in *R. prolixus* was highlighted in a recent study (Vieira et al., 2018).

In this context, the present work aims to investigate the influence of ecdysone on the regulation of the expression of the AMPs genes *Defensin A*, *Defensin B*, *Defensin C,* and Prolixicin, as well as the canonical components of Toll and IMD signaling pathways, *RpDorsal* and *RpRelish*, respectively, in *R. prolixus*, as well as their influence on intestinal microbiota homeostasis.

MATERIAL AND METHODS

Rhodnius prolixus maintenance and ethics statement

All experiments were undertaken with *R. prolixus* 5th instar nymphs reared and maintained at the Laboratório de Bioquímica e Fisiologia de Insetos IOC/FIOCRUZ at a relative humidity of 50–60% and at 27°C (Azambuja, 1997) After molting, insects were randomly chosen, starved for 15–20 days, and then fed with defibrinated rabbit blood through a membrane feeding apparatus (Azambuja, 1997). The *Instituto de Ciência e Tecnologia em Biomodelos* (ICTB) provided the rabbit blood used in all experiments, in agreement to the Ethical Principles in Animal Experimentation and accepted by the Comissão de Ética no Uso de Animais do Instituto Oswaldo Cruz (CEUA/FIOCRUZ, under the protocol number LW019/17).

Insects oral treatment

R. prolixus 5th instar nymphs were fed with blood containing AZA (Sigma) and α-ecdysone (Sigma), both dissolved in 1:4 ethanol–saline in final concentrations of 1 and 2.5 µg/ml, respectively. The control groups were fed with blood containing the same final concentration of solvent used to prepare the compounds.

Analysis of *R. prolixus* gene expression by RT-qPCR

The relative expression of *R. prolixus* antimicrobial peptides genes (*defA, defB, defC,* and *prol*) and the transcription factors *RpDorsal* and *RpRelish* (from Toll and IMD pathways, respectively) were investigated by reverse transcription-quantitative PCR (RT-qPCR). On different days after feeding, anterior midgut samples were collected from dissected *R. prolixus* 5th instar nymphs (control and treated insects) in three pools containing five anterior midguts each, as previously described in Vieira et al., 2016. Samples were stored at -80ºC until total RNA extraction, which was performed with the NucleoSpin® RNA II Kit (Macherey-Nagel, Düren, Germany). The purified RNA was

quantified in a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Then, the cDNA strand was synthesized with a First-Strand cDNA Synthesis Kit (GE Healthcare Buckinghamshire, UK) using 2.5 μg of total RNA. cDNA amounts were measured in a Qubit Fluorimeter (Life Technologies) with the ssDNA assay kit. The design of primers for *R. prolixus* genes (housekeeping and target genes) were based from previously published cDNA sequences: *α-tubulin* and *GAPDH* (Paim et al., 2012), *defA, defB,* and *defC* (Lopez et al., 2003; Vieira et al., 2016); *prol* (Ursic-Bedoya et al., 2011; Vieira et al., 2016), *RpDorsal* (Ursic-Bedoya et al., 2009), *RpRelish* (Mesquita et al., 2015). Real-time quantitative polymerase chain reactions (RT-qPCR) were conducted with GoTaq® qPCR Master Mix (PROMEGA). Gene expression assays and analysis were performed as described in Vieira et al., 2016, in the ABI PRISM 7500 Sequence Detection System (Applied Biosystems) at the FIOCRUZ facilities (Real-Time PCR Platform RPT-09A). Data were analyzed with the Expression Suite v1.0.3 software (Life Technologies), considering the amplification efficiency of each target and the comparative Ct (ΔΔCt) method (Livak and Schmittgen, 2001).

***Serratia marcescens* analysis**

Anterior midguts from *R. prolixus* 5th instar nymphs were collected from unfed insects and at one, five, and seven days after feeding with non-supplemented blood or blood containing AZA. *S. marcescens* load was quantified by RT-qPCR, using the following pair of primers: Forward 5' GGTGAGCTTAATACGTTCATCAATTG 3'; Reverse 5' GCAGTTCCCAGGTTGAGCC 3' (Saikaly et al., 2007). The relative expression analyses of 16S-rRNA from *S. marcescens* was performed using cDNA from three pools of five anterior midguts from control and treated insects (n=3) in three independent experiments, according to Vieira et al., 2016.

Statistical analysis

Experimental data were analyzed using 1-way ANOVA Student's T, Kruskal-Wallis test, or Mann-Whitney test on GraphPad Prism 5 software. Significance levels of differences between groups are shown in the respective figures and legends. They were considered statistically different when $p < 0.05$.

RESULTS

Effect of oral treatment with azadirachtin on insect molting.

Fifth instar nymphs of *Rhodnius prolixus* were orally treated with blood containing 1µg/mL of AZA, or 1µg/mL of AZA plus 2.5 µg/mL of ecdysone and the molting process was observed. Our results showed that the group of insects treated with AZA had a partial inhibition of ecdysis when compared to the control group (insects fed with blood containing solvent only) (Figure 1, $P<0.01$). This effect of AZA on molting was counteracted by the addition of ecdysone in the insect blood meal (Figure 1, $p < 0.05$). There was no significant difference between control nymphs and insects treated with AZA plus ecdysone (Figure 1).

Components from Toll and IMD signaling pathways were modulated by azadirachtin treatment in the *Rhodnius prolixus* midgut.

The effects of the triterpenoid on the expression of genes related to Toll and IMD signaling pathways in the *R. prolixus* midgut cells were evaluated. Expression of NF-κB transcription factor genes *RpRelish* (IMD pathway transcription factor) and *RpDorsal* (Toll pathway transcription factor) were quantified at different time points by RT-qPCR. Temporal analysis of IMD and Toll pathway transcription factors showed that AZA induced a significant downregulation of *RpRelish* expression in *R. prolixus* anterior midgut at 1 day after feeding (DAF), 5 DAF, and 7 DAF (Figure 2A, $p < 0.001$; $p < 0.05$; $p < 0.05$). The concomitant administration of ecdysone counteracted these inhibitory effects caused by AZA. Insects treated with AZA + ecdysone presented no significant differences in *RpRelish* expression at 1DAF comparing to the control group (Figure 2A). However, a slight increase in *RpRelish* expression was observed in insects treated with AZA + ecdysone at 5 and 7 DAF comparing to the control insects (Figure 2A, $p < 0.05$; $p < 0.05$). Comparing AZA treated with AZA + ecdysone treated insects, the latter group showed a strong upregulation in *RpRelish* mRNA level at 1, 5 and 7 DAF (Figure 2A, $p < 0.001$; $p < 0.05$; $p < 0.01$).

In contrast, AZA treatment resulted in upregulation of *RpDorsal* expression at 5 DAF, compared to the control group (Figure 2B, $p < 0.01$). Later, at 7 DAF, the relative expression of *RpDorsal* in AZA treated-insects was lower than in the control group (Figure 2B, $p < 0.05$). Furthermore, the insects treated with AZA + ecdysone presented the same mRNA levels of *RpDorsal* compared to the control group (Figure 2B).

Ecdysone treatment counteracts the repression of antimicrobial peptides gene expression induced by azadirachtin treatment.

Following the observation of the modulation of NF-κB transcription factor genes, *RpRelish* and *RpDorsal*, in insects fed with AZA, the effect of the triterpenoid was assessed on genes downstream of the immune signaling pathways, by analysis of AMPs gene expression in *R. prolixus* midgut cells (Figure 3 and Figure 4).

Expression of the different antimicrobial peptides genes was quantified through RT-qPCR of nymphs fed with blood, blood containing AZA and blood containing AZA + ecdysone, at 1, 5 and 7 DAF. Treatment of nymphs with AZA inhibited the expression of defensin A (*defA*), defensin B (*defB*) and prolixicin (*prol*), significantly at 1, 5 and 7 DAF when compared to the control insects (Figure 3A; $p < 0.01$; Figure 3B, $p < 0.01$; Figure 3C, $p < 0.001$; Figure 3D, $p < 0.001$; Figure 3E, $p < 0.01$; Figure 3F, $p < 0.05$; Figure 4D, $p < 0.05$; Figure 4E, $p < 0.05$; Figure 4F, $p < 0.001$). In contrast, in AZA treated-nymphs there was an augmentation of defensin C (*defC*) mRNA levels on 7 DAF (Figure 4C; $p < 0.01$) but this effect was not observed at 1 and 5 DAF after oral treatment (Figure 4A; 4B). At 1 DAF, *defC* mRNA levels in AZA treated insects were similar to the control group (Figure 4A) and lower in comparison to controls at 5 DAF (Figure 4B, $p < 0.01$).

The effects of AZA on mRNA levels of *defA* and *defB* were reversed with concomitant treatment with ecdysone, observed at both 5 DAF (Figure 3B, $p < 0.05$; Figure 3E, $p < 0.01$) and at 7 DAF (Figure 3C, $p < 0.05$; Figure 3F, $p < 0.05$). The rescue of *prol* mRNA levels was observed at all time points analyzed, in comparison to AZA treated groups (Figure 4D, $p < 0.05$; Figure 4E, $p < 0.001$; Figure 4F, $p < 0.001$). When *prol* gene expression in AZA + ecdysone group was compared to the control group, a higher mRNA abundance in AZA + ecdysone treated insects at 1 and 5 DAF was observed (Figure 4D, $p < 0.05$; Figure 4E, $p < 0.01$) but not at 7 DAF (Figure 4F).

***Serratia marcescens* analyses: population dynamics and susceptibility to antimicrobial factors from the midgut of *R. prolixus* treated with azadirachtin.**

To explore the broader effects of ecdysone inhibition in *R. prolixus*, the population dynamics of *S. marcescens*, an abundant bacterial species naturally found in the *R.*

prolixus gut, was examined in the insect midgut to understand if the hormone has a role in maintaining gut homeostasis. The analysis of the expression of 16S rRNA of *S. marcescens* demonstrated that bacterial abundance increased in the *R. prolixus* midgut around 2,000-fold one day after blood ingestion when compared with unfed insects (Figure 5A; $p < 0.05$). Also, a peak of bacterial abundance was observed at the fifth day after the blood meal, in comparison with the unfed group (Figure 5A, $p < 0.01$) and insects 1 DAF (Figure 5A, $p < 0.05$).

A significant increase (approximately 3-fold) in the bacterial population was observed in insects fed with blood plus AZA in comparison to untreated control insects at 5 DAF (Figure 5; $p < 0.05$). Nevertheless, at 7 DAF, the bacterial ~~number~~ abundance decreased significantly, reaching approximately 5-fold lower levels than in control insects (Figure 5; $p < 0.01$).

DISCUSSION

Ecdysone is a steroid hormone synthesized by insect prothoracic glands (PG). These glands are stimulated by prothoracicotropic hormone (PTTH), which is secreted from the insect brain (Wigglesworth, 1934a, 1934b). In fifth instar nymphs of *R. prolixus*, ecdysone is released in two distinct pulses. The first one occurs a few hours after a blood-feed. The second ecdysone pulse starts around the first week after feeding (Vafopoulou and Steel, 1989). The primary function of ecdysone is to coordinate the molting process and insect growth (Wigglesworth, 1974; Yamanaka et al., 2013). Previous studies have shown that the natural product azadirachtin (AZA) interferes with the PTTH-ecdysone pathway, affecting the insect molt (Chaudhary et al., 2017; Garcia et al., 1990; Gonzalez and Garcia, 1992; Vafopoulou and Steel, 1989). The effect of AZA on *R. prolixus* ecdysis has been associated with the interference in the release of PTTH by neurosecretory cells - leading to a blockage in ecdysone synthesis and release by the PGs (Garcia et al., 1987). Moreover, by interacting directly with the ecdysone receptor (EcR) as an ecdysone antagonist, AZA can also impair hormone function (Oliveira, 2019). In the present work, we observed a significant reduction of ecdysis in fifth instar nymphs of *R. prolixus* orally treated with AZA. The inhibitory effect of AZA was counteracted through the simultaneous treatment of insects with ecdysone, as seen by previous studies (Albuquerque-Cunha et al., 2004; Gonzalez and Garcia, 1992).

Here, oral treatment of *R. prolixus* nymphs with AZA caused an immediate decrease in expression of the IMD transcription factor *RpRelish* gene. This effect was reversed by the addition of exogenous ecdysone in the insect blood meal, suggesting that the hormone regulates the IMD pathway. Likewise, it has been suggested that the IMD pathway is under hormonal regulation in *Drosophila* (Flatt et al., 2008; Rus et al., 2013; Zheng et al., 2018) and in *Locusta migratoria* (Han et al., 2020; Han et al., 2017), in which ecdysone activates the expression of genes encoding a peptidoglycan recognition protein LC (PGRP-LC). Genomic and transcriptomic analyses highlighted the presence of some, but not all, genes encoding components of the IMD pathway in *R. prolixus* (Nishide et al., 2019; Ribeiro et al., 2014; Salcedo-Porras et al., 2019; Zumaya-Estrada et al., 2018). In this context, an increase in the expression of *RpRelish* was previously associated with the synthesis of some antimicrobial peptides (Vieira et al., 2018). Moreover, a reduced level of AMPs gene expression was detected after silencing *RpRelish* by RNA interference (da Mota et al., 2018; Salcedo-Porras et al., 2019).

The decrease in *RpRelish* expression after AZA treatment became less significant on each subsequent day after treatment. Nevertheless, on the seventh day, a significant difference was detected in comparison with the control group treated simultaneously with AZA and ecdysone, indicating that exogenous ecdysone could, in this case, be directly acting on upregulation of the gene. Consequently, the impact of AZA on *RpRelish* expression appears to be related to the first peak of ecdysone release by the prothoracic glands.

Ecdysone can activate the expression of different antimicrobial peptides in insects (Ma et al., 2019; Wang et al., 2014; Zanarotti et al., 2009), independent of microbial challenge (Mai et al., 2017). This priming effect of ecdysone on the innate immune response was also reported in the *Anopheles gambiae* (Reynolds et al., 2020). The hormone induces the upregulation of some AMP genes and reduces *Plasmodium berghei* and bacterial survival in mosquitoes (Reynolds et al., 2020). Together, this indicates that endogenous ecdysone can regulate *RpRelish* and thereby trigger a humoral immune response in *R. prolixus*. Coordinating this immune response with ecdysis is likely to be essential for protection against pathogens during molting when the insects are vulnerable to infection.

In contrast, AZA did not affect the expression of *RpDorsal* (the Toll transcription factor) on the first day after treatment. However, later on, five days after feeding, the insects treated with AZA showed an upregulation of *RpDorsal* levels almost eight times

higher than in control insects. Surprisingly, in the AZA-treated group, *RpDorsal* expression declined dramatically by the seventh day after feeding, remaining at levels below those observed in control insects. These results indicate a different pattern of hormonal modulation on both Toll and IMD pathways in *R. prolixus*. Moreover, concomitant treatment with ecdysone did not reverse the effects of AZA on *RpDorsal* expression, suggesting a different indirect regulatory mechanism compared to that of *RpRelish*.

Previous studies demonstrated that AZA treatment could inhibit *T. cruzi* development by an indirect effect by disrupting the perimicrovillar membrane structure and the epithelial cells of the midgut (Gonzalez and Garcia, 1992; Gonzalez et al., 1999). This effect is counteracted by concomitant treatment with ecdysone, turning the intestinal lumen into a proper environment for epimastigote adhesion to the midgut epithelial cells, a significant event in the parasite life cycle (Alves et al., 2007; Gonzalez et al., 1999). The effects of ecdysone on the regulation of expression of AMP genes, as has been reported in other insects (Ma et al., 2019; Reynolds et al., 2020; Rus et al., 2013), is another way the hormone may impact *T. cruzi* infection. The treatment of *R. prolixus* nymphs with AZA resulted in a reduction of the expression of both the *DefA* and *DefB* genes, mirroring the inhibition of *RpRelish*. (Vieira et al., 2018) observed a decrease in *DefA* and *DefB* mRNA levels as a consequence of the inhibition of *RpRelish* in the *R. prolixus* midgut. (Salcedo-Porras et al., 2019) also reported a decrease in the *DefA* transcription levels in *RpRelish* knockdown *R. prolixus* challenged with the Gram-negative bacteria *Enterobacter cloacae. DefA* is upregulated in the midgut of *R. prolixus* infected with *S. aureus* (Vieira et al., 2014), with evidence that this AMP has a role against Gram-positive bacterial infections. Together, these results support the function of the IMD pathway in the regulation of *DefA* and *DefB* in *R. prolixus*.

Furthermore, AZA treatment also induced downregulation in the levels of another AMP, prolixicin (*Prol*), with ecdysone treatment reversing this effect, mirroring the impact on *RpRelish* expression. These results support those observed by (Salcedo-Porras et al., 2019) and (Vieira et al., 2018), showing that the suppression of *RpRelish* in *R. prolixus* induces downregulation of *Prol* expression (Salcedo-Porras et al., 2019).

In contrast, *DefC* expression was not immediately affected by AZA treatment. Whereas *DefA* and *DefB* are closely related in their sequences, *DefC* diverges a little from the other two defensins (Lopez et al., 2003; Waniek et al., 2009). Moreover, infection of *R. prolixus* with either *T. rangeli* or *T. cruzi* induces *DefC* upregulation in

the insect midgut while the expression of *DefA* and *DefB* remains unchanged or diminished (Vieira et al., 2015; Vieira et al., 2016). Besides, previous analyses of the effects knockdown of *RpRelish* indicated no impact of the IMD pathway on expression *DefC* (Salcedo-Porras et al., 2019). These results emphasize dissimilarities in the regulation of the different *R. prolixus* AMPs. Similar to *DefC* regulation, the expression of *RpDorsal* and the size of the *S. marcescens* gut population were not affected by AZA treatment at 1 DAF. Still, it is important to note that blood ingestion induces a massive proliferation of the *S. marcescens* population (Fig 5A). However, on the 5th day after AZA treatment, a significant reduction in the transcription level of *DefC* was noted, while the *S. marcescens* load had increased in the insect midgut. The dynamic interplay between the immune response and the resident microbiota is highlighted by this initial proliferation of *S. marcescens* in AZA-treated insects with a reduced expression on AMPs until 5 DAF. The enhancement of *S. marcescens* load in AZA treated insects may, in turn, further modulate the Toll pathway, evidenced by an increase in *Rpdorsal* expression at this time point. Seven days after AZA treatment, a massive increase (almost 200-fold) in *DefC* expression was observed. The higher expression of this AMP correlated with a significant decrease in the population of the commensal bacterium at this time point, likely reflecting the antimicrobial activity of DefC. The effect on the bacterial load, as a direct or indirect consequence of hormonal disruption of gut homeostasis, suggests a stimulus of DefC synthesis, likely triggered by bacterial proliferation. This feedback mechanism could play an essential role in population control of *S. marcescens* in the digestive tube of *R. prolixus*, as suggested previously (Vieira et al., 2015; Vieira et al., 2018; Vieira et al., 2016).

Since the *R. prolixus* midgut is the primary organ in the interface between foreign microorganisms and insect immune responses, the fast activation of IMD and Toll pathways may be critical to regulate variation in the populations of acquired or already established microbes, which are also impacted by blood ingestion. Here we describe the impact of the neuroendocrine system in the maintenance of gut homeostasis, through the modulation of *R. prolixus* immune signaling pathways, in this complex environment represented by the insect midgut. The dynamics of the immune response to various microorganisms in this niche deserve further investigations.

CONCLUSIONS

AZA inhibits the release and function of ecdysone in *R. prolixus*, a vector of *T. cruzi.* We show that AZA also inhibits expression of both a critical IMD-pathway transcription factor, *RpRelish*, and several AMP genes, effects that can be reversed by concomitant treatment with ecdysone. AZA inhibition of the IMD pathway disrupts gut microbial homeostasis, resulting in an increased abundance of the commensal bacterium *S. marcescens*. This imbalance may explain subsequent upregulation of the Toll pathway and *DefC*, encoding another AMP, with consequent suppression of the *Serratia* population. The study highlights aspects of the regulation of immune responses in *R. prolixus* important for maintaining gut microbial homeostasis.

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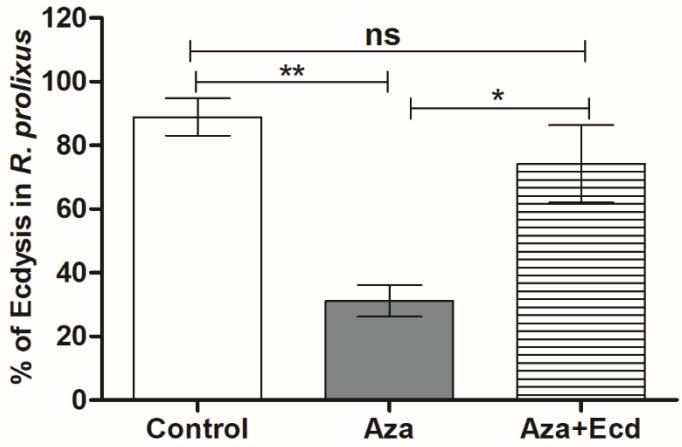

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772

773 **Figure 1. Effects of azadirachtin and ecdysone treatment on the moulting of**
774 ***Rhodnius prolixus*.** *R. prolixus* 5th instar nymphs were previously fed with blood
775 containing solvent (control; white column), azadirachtin [1µg/ml] (grey column) and
776 azadirachtin [1µg/ml] plus ecdysone [2.5 µg/ml] (striped column). Insect molting was
777 monitored up to 45 days after the blood meal. Bars represent the mean ± SEM of three
778 independent experiments (n=15). Means were compared using Student's T-test;
779 ** $p < 0.01$, * $p < 0.05$.

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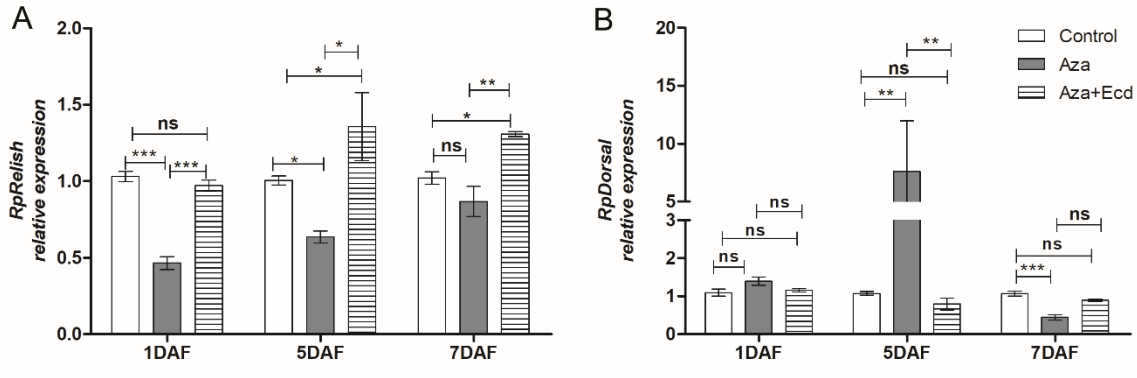

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782 **Figure 2: Azadirachtin modulates genes related to immune signaling pathways in**
783 ***Rhodnius prolixus* midgut.** Expression of Relish (*RpRelish*), the IMD pathway
784 transcription factor, and Dorsal (*RpDorsal*), the Toll pathway transcription factor, was
785 analyzed in anterior midguts of 5th instar nymphs of *R. prolixus* at different days after
786 insect feeding (DAF) on blood containing solvent (white column - control) or azadirachtin
787 [1µg/ml] (grey column - Aza) or azadirachtin [1µg/ml] plus ecdysone (2.5 µg/ml) (striped
788 columns - Aza+Ecd). Data were quantified using the gene expression of control insects

as the calibrator shown as the relative expression of (A) *RpRelish*, (B) *RpDorsal* on the 1, 5 and 7 days after feeding (DAF). Bars represent the mean ± SEM of 3 independent experiments with three pools of insects (n=3). Means were compared using Student's T-test; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

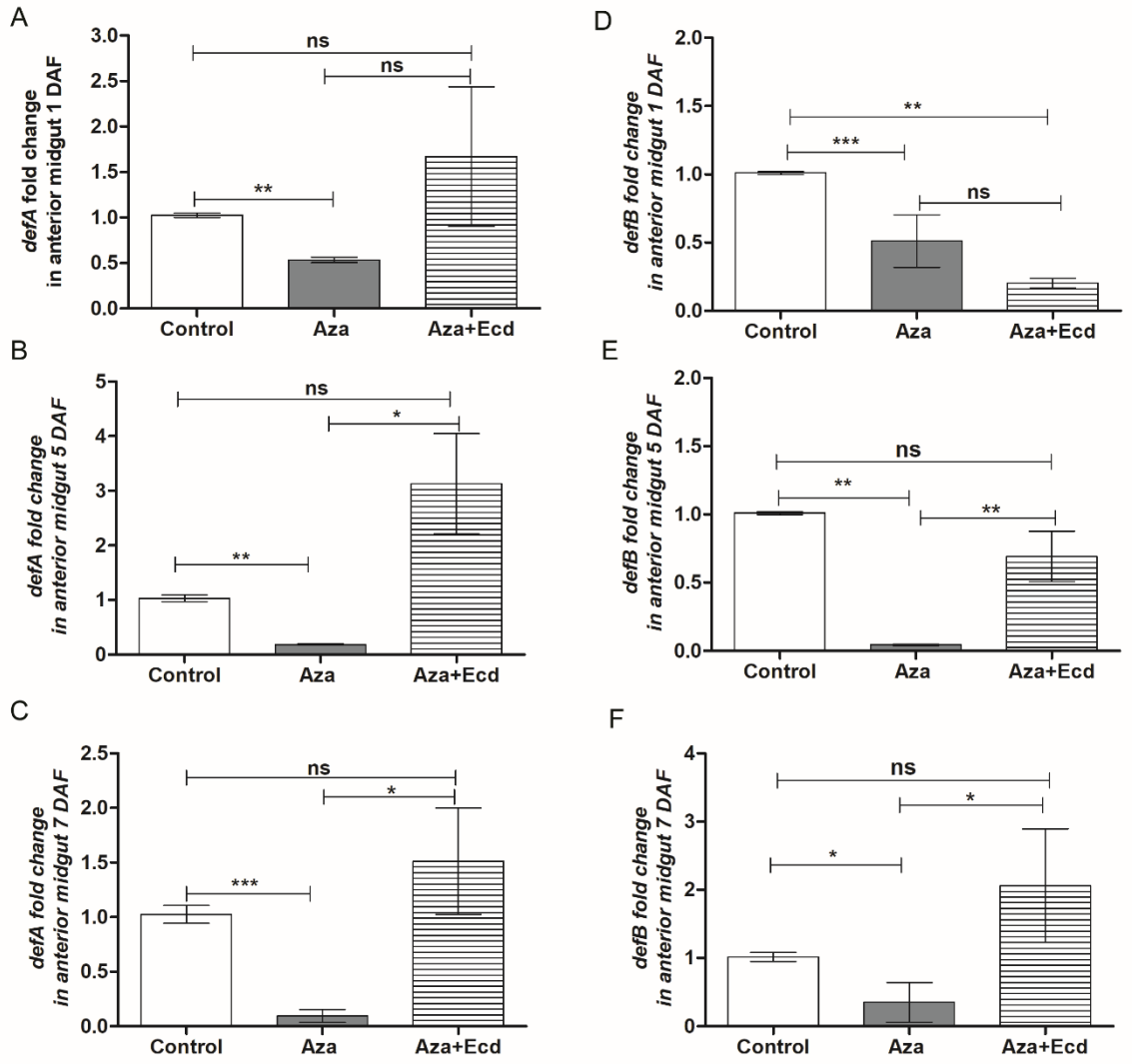
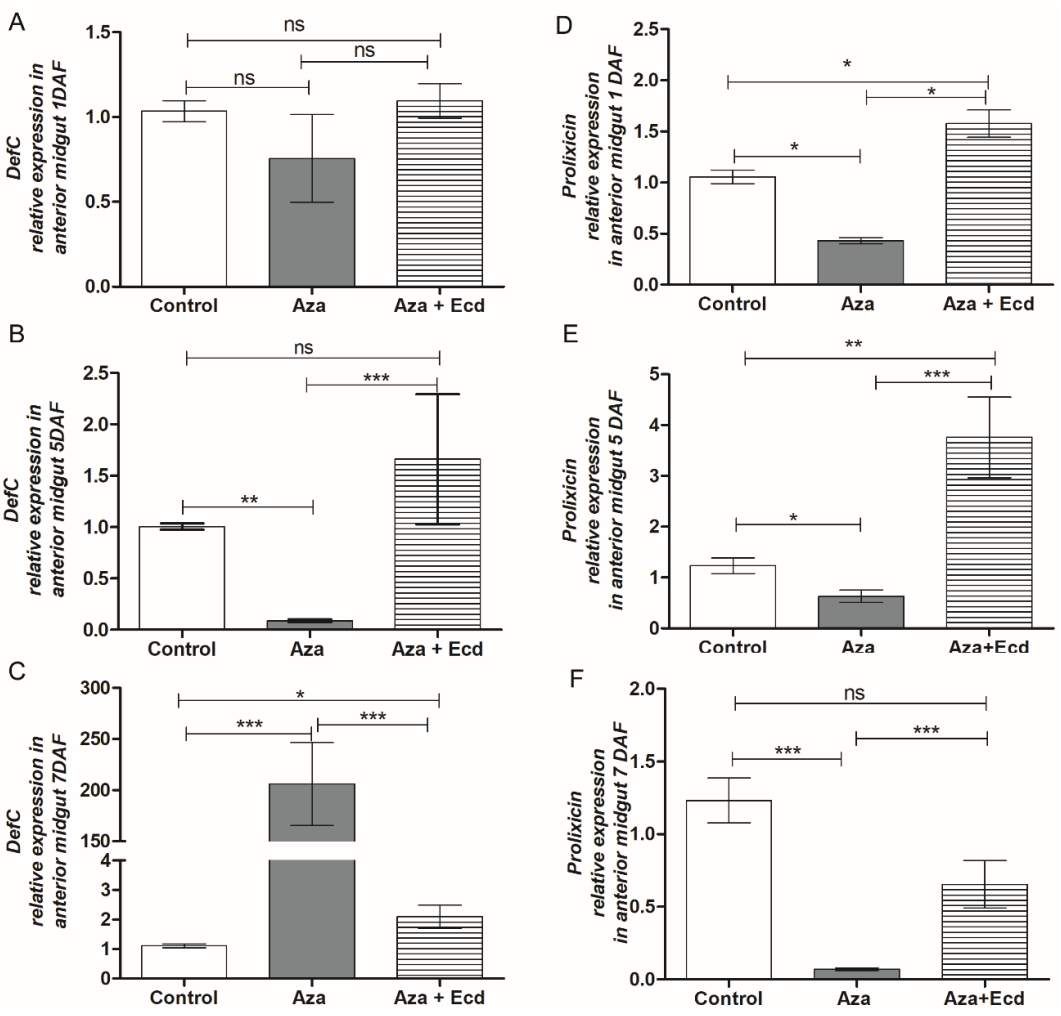


Figure 3. Ecdysone treatment counteracts defensin A and defensin B gene expression of *Rhodnius prolixus* anterior midgut. *R. prolixus* 5th instar nymphs were previously fed with a blood containing: solvent (white column - control), azadirachtin [1µg/ml] (grey column-Aza) or azadirachtin [1µg/ml] plus ecdysone [2.5 µg/ml] (striped column-Aza + Ecd). Anterior midgut samples were collected 1, 5 and 7 days after feeding (DAF). Data were quantified using the gene expression of control insects as the calibrator shown as the relative expression of *defA* (A, B, C), *defB* (D, E, F) on the 1, 5 and 7 DAF. Bars represent the mean ± SEM of 3 independent experiments with three pools of insects (n=3). Means were compared using Student T-test; *** $p < 0.001$, **$P<0.01$, * $p < 0.05$.



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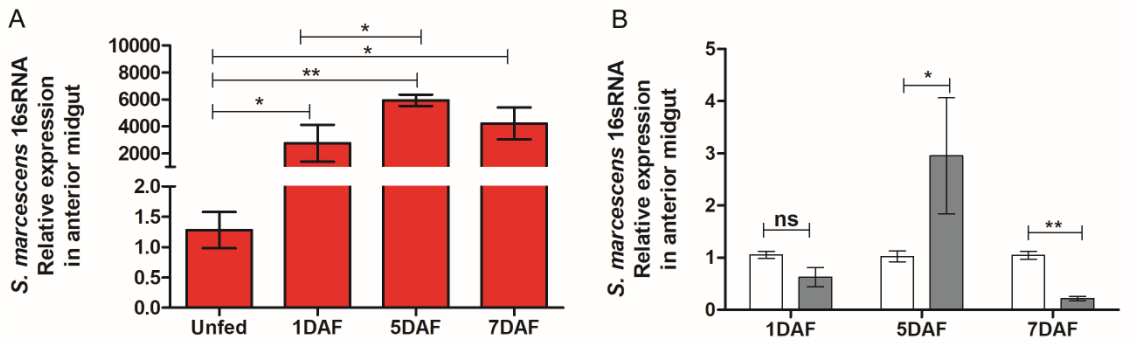
805 **Figure 4. Ecdysone treatment counteracts defensin C and Prolixicin gene expression**
806 **of *Rhodnius prolixus* anterior midgut.** *R. prolixus* 5th instar nymphs were previously
807 fed with a blood containing: solvent (white column - control), azadirachtin [1µg/ml] (grey
808 column-Aza) or azadirachtin [1µg/ml] plus ecdysone [2.5 µg/ml] (striped columns-Aza
809 + Ecd). Anterior midgut samples were collected 1, 5 and 7 days after feeding (DAF). Data
810 were quantified using the gene expression of control insects as the calibrator shown as
811 the relative expression of *defC* (A, B, C), *Prol* (D, E, F) on the 1, 5 and 7 DAF. Bars
812 represent the mean ± SEM of 3 independent experiments with three pools of insects (n=3).
813 Means were compared using Student T-test; *** $p < 0.001$, **$P<0.01$, * $p < 0.05$.



814

815 **Figure 5– Effect of azadirachtin on *Serratia marcescens* load in *Rhodnius prolixus***
816 **anterior midgut.** *R. prolixus* 5th instar nymphs were previously fed with blood containing
817 solvent or azadirachtin [1µg/ml]. Determination of bacterial load in *Rhodnius prolixus*
818 anterior midgut was performed in unfed insects and at 1, 5 and 7 days after feeding (DAF)
819 through the analysis of relative expression of 16S-rRNAs gene from *Serratia marcescens*
820 by RT-qPCR. All data were normalized to the *R. prolixus* 18S-rRNA. A – Bacterial load
821 in *R. prolixus* before and after a blood meal. B- *S. marcescens* abundance in control (white
822 column) and azadirachtin treated insects (grey column); values plotted relative to control
823 values. Bars represent the mean ± SEM of 3 independent experiments with three pools
824 of insects ($n = 3$). Means were compared using Student's *T*-test; ** $p < 0.01$, * $p < 0.05$,
825 ns indicates a non-significant difference.

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