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IMPACTS OF PARASITES ON INSECT MOULTING

VIÇOSA, MINAS GERAIS

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Thesis submitted to the Entomology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Simon Luke Elliot

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ABSTRACT

QUEIROZ, Elenir Aparecida, D.Sc., Universidade Federal de Viçosa, March, 2023. **Impacts of parasites on insect moulting**. Adviser: Simon Luke Elliot. Co-advisers: Alessandra Aparecida Guarneri and José Eduardo Serrão.

Insect development occurs through moulting, where the individual generates a new cuticle and sheds the old one, allowing the insect to grow and develop. This process can be impacted by parasites. However, the way different parasites can impact insect moulting is unknown. We first review studies on insect infection and its effect on insect moulting by considering interactions between insects and: (i) protozoans; (ii) fungi; (iii) viruses; and (iv) parasitoids. We show that insect moulting is impacted by moulting delays, non-moult and defects after moulting, besides increasing insect mortality during moulting. However, while there is evidence that parasite-infected insects can experience increased mortality during moulting, this process is not well understood yet. We then documented this so as to raise hypotheses about the mechanisms, with a focus on behaviour, using as a model system the triatomine bug *Rhodnius prolixus* (Hemiptera: Reduviidae) and the protozoan parasite *Trypanosoma rangeli*. We quantified mortality and investigated alterations in ecdysis-related behaviour. We found that the high mortality of infected insects is related to increases in the time taken to finish ecdysis. We then investigated cuticular thickness and ecdysone levels as potential factors to explain this phenomenon. Mortality of infected insects was 6% higher than that of uninfected insects. Infected insects show a thinner cuticle over time and lower ecdysone levels compared to controls. We suggest this thin cuticle in infected insects is associated with the moulting delay. As moulting is delayed, we investigate glycogen content in the fat body and intestine morphology as possible factors. Infected insects have low glycogen content in the fat body and intestine morphology is not altered between infected and control insects, but intestine epithelium is apparently thicker in infected than the control. Our results allow us to understand better the effect of insect infections at behavioural, morphological and hormonal levels. This study is a starting point for further reasearch on the moulting physiology of insects infected by parasites in order to improve our understanding of insect-parasite interaction.

Keywords: Cuticle. Development. Ecdysis. Ecdysone. Fungus. Moult. Parasitoid. Pathogen. Physiology. Virus.

RESUMO

QUEIROZ, Elenir Aparecida, D.Sc., Universidade Federal de Viçosa, março de 2023. **Impactos de parasitas na muda dos insetos**. Orientador: Simon Luke Elliot. Coorientadores: Alessandra Aparecida Guarneri e José Eduardo Serrão.

O desenvolvimento dos insetos ocorre por meio de muda, onde o indivíduo gera uma nova cutícula e se libera a antiga, permitindo que ele cresça e se desenvolva. Este processo pode ser afetado por parasitas. No entanto, a forma como diferentes parasitas podem impactar a muda dos insetos é desconhecida. Primeiro revisamos estudos sobre infecção por insetos e seu efeito na muda de insetos, considerando as interações entre insetos e: (i) protozoários; (ii) fungos; (iii) vírus; e (iv) parasitóides. Mostramos que a muda dos insetos é impactada por atrasos na muda, não muda e defeitos após a muda, além de aumentar a mortalidade dos insetos durante a muda. No entanto, embora existam evidências de que os insetos infectados com parasitas podem sofrer um aumento da mortalidade durante a muda, este processo ainda não é bem compreendido. Então, documentamos isso para levantar hipóteses sobre os mecanismos, com foco no comportamento, usando como sistema modelo o inseto triatomíneo *Rhodnius prolixus* (Hemiptera: Reduviidae) e o parasita protozoário *Trypanosoma rangeli*. Quantificamos a mortalidade e investigamos alterações no comportamento relacionado à ecdise. Descobrimos que a alta mortalidade de insetos infectados está relacionada ao aumento do tempo necessário para terminar a ecdise. Em seguida, investigamos a espessura cuticular e os níveis de ecdisona como fatores potenciais para explicar esse fenômeno. A mortalidade de insetos infectados foi 6% maior que a de insetos não infectados. Os insetos infectados apresentam cutícula mais fina ao longo do tempo e níveis mais baixos de ecdisona em comparação ao controle. Sugerimos que tal cutícula fina em insetos infectados esteja associada ao retardo da muda. Como a muda é atrasada, investigamos o conteúdo de glicogênio no corpo gorduroso e a morfologia do intestino como possíveis fatores. Os insetos infectados têm baixo teor de glicogênio no corpo gorduroso e a morfologia do intestino não é alterada entre os insetos infectados e o controle, mas o epitélio intestinal é aparentemente mais espesso nos infectados do que no controle. Nossos resultados nos permitem compreender melhor o efeito das infecções por insetos a níveis comportamental, morfológico e hormonal. Este estudo é um ponto de partida para futuras pesquisas sobre a fisiologia da muda de insetos infectados por parasitas, melhorando a nossa compreensão da interação inseto-parasita.

Palavras-chave: Cutícula. Desenvolvimento. Ecdise. Ecdisona. Fungo. Muda. Parasitoide. Patógeno. Fisiologia. Vírus.

SUMMARY

1. GENERAL INTRODUCTION

Growth is an important part of the developmental history of an insect, which goes from egg to adult. Insect growth is discontinuous as the rigid cuticle limits body expansion. Thus, insects increase in size by moulting, which is the periodic formation of a new cuticle that has surface area greater than the previous cuticle, followed by ecdysis (the shedding of the old cuticle) (Gullan and Cranston, 2014). By moulting, the insect body increases in size saltatory way (Klowden, 2013). Moulting success is critical for an insect as it can increase its susceptibility to predators, pathogens or environmental conditions – as well as to cause problems of organs or structure formation, if the regulation of moulting is affected somehow (Klowden, 2013).

In this thesis we aim to further our understanding of why diseased insects may have high mortality during moulting. We look for morphological, behavioural or hormonal factors that can help explain this phenomenon. In this context, for this General Introduction, we first describe the hormonal regulation of insect moulting as our study must be based on an understanding of the physiology of the process. Secondly, we show examples of effects of parasites on insect moulting, looking for possible patterns. Thirdly we introduce the model system used in this thesis. Finally, we give an overview of the thesis.

1.1. Hormonal regulation of moulting

The hormonal regulation of moulting is briefly presented here. More details can be found in Gullan and Cranston (2014) and Klowden (2013). After a stimulus, such as abdominal distension after blood feeding, the insect starts the process of moulting. Hormones stimulate the mitotic division of the epidermal cells, leading to increases in cell volumes and surface area of the epidermis. The process of moulting is initiated by retraction of epidermal cells from the inner surface of the old cuticle; the separation of the old cuticle from the new cuticle is called apolysis, which is incomplete as muscles and sensory nerves mantain their connection with the old cuticle for a while. The subcuticular space formed after apolysis becomes filled with an inactive moulting fluid that has chitinolytic and proteolytic enzymes. These enzymes are activated only when the protective outer layer of the new cuticle is secreted by the epidermal cells. Then, the endocuticle (the inner part of the old cuticle) is digested and probably resorbed, while the new cuticle continues to be deposited as a procuticle.

The last part of the moulting is ecdysis, the release of the old cuticle. Ecdysis begins when the old cuticle breaks down along the dorsal midline; this occurs because of hormone activities and hemolymph pressure. The exuvium (the discarded epicuticle and exocuticle) consists of indigestible proteins, lipids, and chitin. The newly hatched insect expands the new cuticle by swallowing air or water and/or increasing hemolymph pressure. After this expansion, the body surface become sclerotized to form the exocuticle (Fig.1). The time insects spend on sclerotizing their cuticle possibly varies according to the insect group. In holometabolous insect larvae, the exocuticle is restricted to the head capsule; most of the body cuticle remains membranous. After ecdysis, the epidermal cells secrete the endocuticle, which continue to be deposited during the intermoult period. Often the exocuticle (the outer part of the cuticle) is covered with secretion of wax. After the intermoult period, a hormonal stimulus is generated and mitotic division of the epidermal cells initiates all over again in the brain.

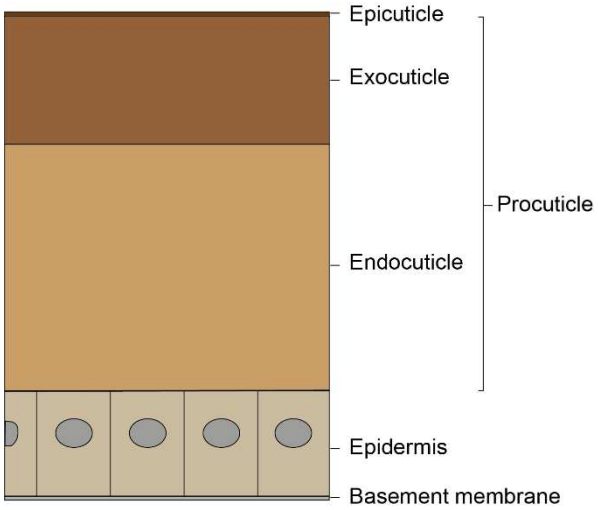

Fig. 1. Scheme of a general structure of insect cuticle. A cuticle structure is composed of a basement membrane, epidermis cells, exocuticle and epicuticle. During moulting, the endocuticle (the inner part of the old cuticle) is digested and resorbed, while the new cuticle is being deposited – in this case it is called procuticle.

A classic view of hormonal regulation of moulting between larval/nymphal stages is represented here (Fig. 2). There are three main types of hormones that control moulting and they are: (i) neuropeptides (e.g. prothoracic tropic hormone - PTTH), ecdysis-triggering

hormones (PETH, ETH) and hatching hormone (EH); (ii) ecdysteroids; and (iii) Juvenile hormone (JH). In the brain, neurosecretory cells secrete PTTH. The PTTH stimulates each moult by synthesizing and secreting ecdysteroids (e.g., ecdysone) by the prothoracic glands. The release of ecdysteroids causes division of the epidermal cells, producing a new cuticle. Immature characteristics are mantained during moulting because of the regulation of JH, which is produced by the corpora allata. JH inhibits adult traits: a high level of JH in the hemolymph is related to a moulting between two larval stages.

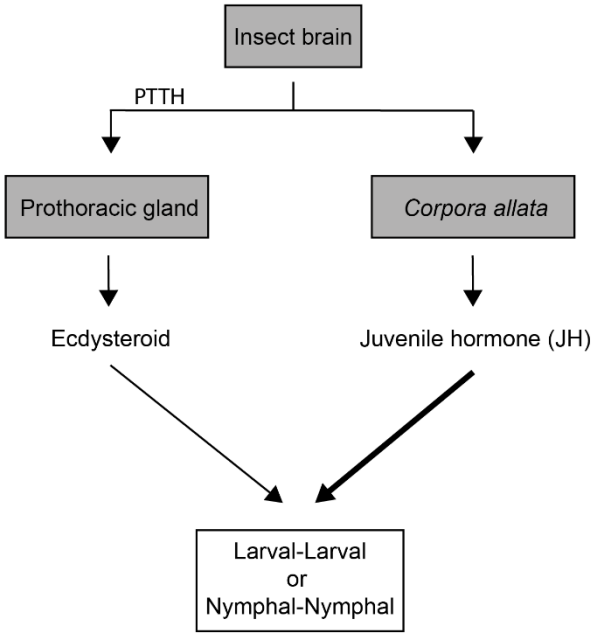

Fig. 2. Simplified schematic diagram of the endocrine control of moulting between larval or nymphal stages in the development of an insect. The thicker the arrow, the higher the hormone level.

Other important moulting hormones are the eclosion hormone (EH) and ecdysis-triggering hormone (ETH), which mediate ecdysis. EH stimulates steroid-enhanced central nervous system to coordinate motor activities associated with the old cuticle shedding. The more recently discovered ETH (in *Manduca sexta*) is as important for moulting as EH, triggering ecdysis. PETH initiates pre-ecdysis behaviour that loosens the muscle attachments of the ecdysis and the ETH stimulates the release of EH from the brain. Moreover, there is a decrease in the level of ecdysteroids, near the end of each moult - this appears to be essential for the ecdysis.

1.2. Insect-parasite interactions: possible reasons for parasites affecting insect moulting

Interactions between insect and parasite can have negative effects on insect moulting, which are cited here. Some studies have described ecdysis-related behaviours, and we expect them to change when an insect is infected. This description is therefore important to make comparisons, and it is a crucial moment in all insect life, because each ecdysis-related behaviour has to occur properly, if not, the insect may not survival during ecdysis, and die in the end of moulting (Klowden, 2013). Generally, the ecdysial behaviour can be divided into distinct phases, which have been characterized in insects such as crickets (Carlson, 1977), locusts (Hughes, 1980), and triatomines (Ampleford and Steel, 1981). Some specific hormones participate in shedding of the old cuticle (Chapman, 2013; Klowden, 2013), and infection by pathogenic microorganisms may contribute to the insect not shedding its old cuticle adequately. For instance, swallowing air as well as tracheal inflation prior to or during ecdysis are essential factors to exert pressure on and split the old cuticle (Reynolds, 1980; Zitnan et al., 2012). Additionally, in the interaction between *R. prolixus* and *T. rangeli*, it has been reported that tracheal cells are damaged in infected insects (Watkins, 1971), which possibly impairs these insects from getting the air necessary to increase the volume of the body. Moreover, if a pathogen infects an insect, it can cause an impact on other insect biological process, or yet, impact symbionts in the insect gut, as *Nocardia rhodnii* in *Rhodnius prolixus* (Watkins, 1971).

Some insect pathogens can inhibit moulting of their hosts through a possible inactivation of the host ecdysteroid hormones (Zhu et al 2021); or the pathogen can use these enzymes to maintain a good physiological condition to grow (Cory et al., 2001). Also, insects such as grasshoppers (Orthoptera) and triatomines (Hemiptera) infected with parasites show increases in their nymph developmental time and mortality (e.g., Elliot et al., 2015; Rodrigues et al., 2016). Moreover, evidence suggests the participation of ecdysone (the moulting hormone) in regulating the immune system in insects (Ahmed, et al 1999; Azambuja et al., 2017) and phagocytic activities of the hemocytes (Dimarcq et al 1997; Figueiredo et al 2006). Also, ecdysone has a role on antimicrobial peptides (AMPs) synthesis in *Drosophila* (e.g., Rus et al. 2013). Thus, ecdysone levels may be impacted when a microorganism is present, as it can be involved in insect defence and, so, survival.

Interestingly, when the caterpillar *Trichoplusia ni* is parasitized by the parasitoid wasp *Hyposoter exiguae*, caterpillar development is delayed and this may be related to the

degradation of juvenile hormone by the activity of juvenile hormone esterase (Thompson, 1982). The regulation of the hosts development and the prevention of the following stages can be a suitable environment for parasite development. Moreover, baculoviruses can manipulate their insect hosts in a variety of ways to facilitate their propagation. A gene that has been extensively studied in this group is the *egt*, and many baculoviruses possess an *egt* gene (Cory et al., 2004). It is known that this gene can impact ecdysteroids (O'Reilly, et al., 1998), and so insect moulting.

1.3. *Rhodnius prolixus* and *Trypanosoma rangeli* as a model system

Rhodnius prolixus and *Trypanosoma rangeli* are used as experimental models in many studies, *R. prolixus* being widely used since it is one of the best-known species from a physiological point of view (Wigglesworth, 1934, 1940; Guarneri and Lorenzo, 2021). This insect is easily maintained in laboratories and survives months of starvation, while moulting can be stimulated by feeding to repletion, a useful experimental consideration (Wigglesworth, 1934; Azambuja et al 2017). Meanwhile, the parasite *T. rangeli* is of value as an experimental model – together with *R. prolixus* – as it infects the insect similarly to other insect pathogens that cross a barrier (in this case, the epithelium of the gut) and infect the haemolymph. So, our results could be informative for considerations of other groups of microorganisms.

Triatomines are hematophagous insects of the family Reduviidae, subfamily Triatominae. To complete development, the five nymphal stages and the adult stage need to feed on blood (Fig. 3). Many species of these blood-sucking insects can act as vectors of Chagas disease (Dias, 2016), and they can transmit *T. cruzi* during their entire life cycle when infected (Zeledon and Rabinovich, 1981). The insect's sanitary risk is directly related to their ability to adapt and survive in contact with humans, besides showing the highest parasitic prevalences compared to other species of triatomines in endemic areas (Schofield, 1985). Triatomines may ingest the parasites from humans by a bite, but these insects can also be infected with trypanosomes from reservoir animals. Moreover, triatomines can survive without feeding for a long time, and also remain for long periods without being dehydrated (Friend and Smith, 1985). That is possible due to their capacity to actively search for microclimate conditions depending on their physiological state (Schilman and Lazzari, 2004).

Rhodnius prolixus can be infected by *T. rangeli*. When this insect feeds on *T. rangeli*-infected mammals, parasite development starts: the trypomastigote forms, ingested with the

blood meal, reach the midgut and differentiate into epimastigotes, becoming able to colonize the entire intestinal tract and potentially cross the intestinal epithelium reaching the haemolymph; in the haemolymph, the parasites multiply and migrate to the salivary glands, where epimastigotes differentiate into metacyclic trypomastigote forms; this form will be transmitted to the vertebrate host through the next insect bite (Fig. 4) (Tobie, 1970; Hecker et al., 1990).

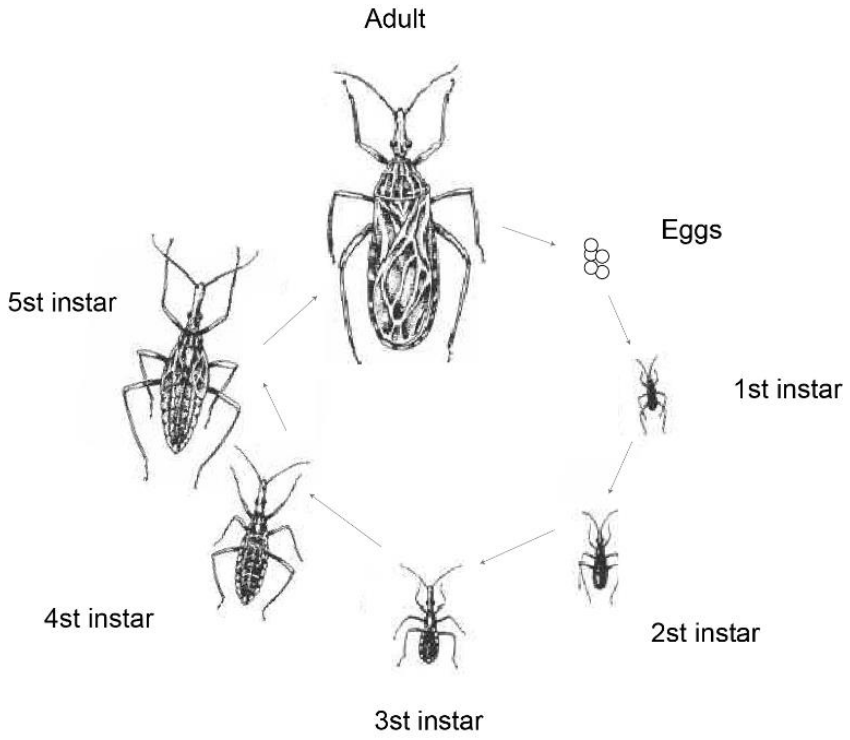

Fig.3. Example of phases of the development of the hemimetabolous insect, *Rhodnius prolixus*. It shows the eggs, five instar nymphs and adult. Adapted from Beaty (1996).

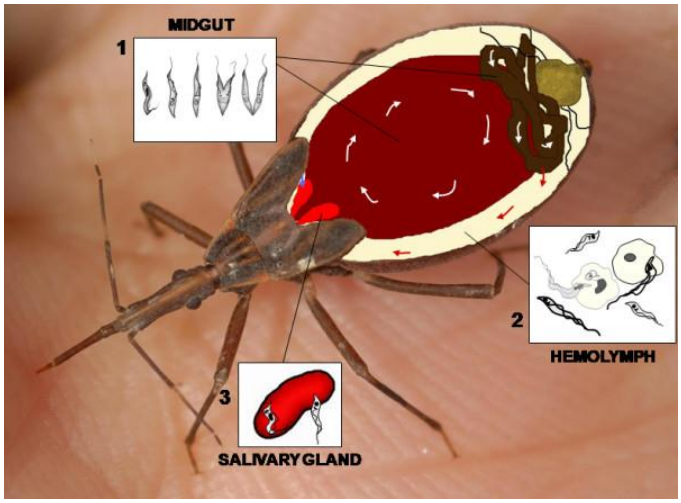

Fig. 4. Life cycle of *T. rangeli* and *R. prolixus*. The *T. rangeli* has several developmental stages in insect vectors and mammalian hosts. *Trypanosoma rangeli* has three main forms: amastigote, epimastigote, and trypomastigote. The triatomine ingests trypomastigote forms during a blood feeding in infected mammals (1). The epimastigote forms can cross the intestinal epithelium to the haemolymph (2). In the haemolimph they multiply and invade the insect salivary glands (3). In these glands, the microorganisms transform into the infective forms. Figure from Garcia et al. (2009).

2. OVERVIEW OF THE THESIS

This thesis has three chapters. In the Chapter One, we present a review of how different groups of pathogenic microorganisms can impact insect moulting, and if such impacts could be a pattern found in the insect pathogen interaction. Particularly, we review studies on insect infection and its effect on the insect moulting process. For that we consider interactions between insects and (i) protozoans; (ii) entomopathogenic fungi; (iii) viruses; and (iv) parasitoid wasps. We discuss these interactions in the contexts of moult and survival of insect hosts. Then, we discuss the mechanistic aspects of this interaction and how these negative effects could be adaptive for pathogens and/or insects. Some suggestions for future research are given.

In Chapter Two, we present an empirical study in which we quantify insect mortality during moulting and consider the hypothesis that insect behaviour during ecdysis is altered due to the pathogen infection. To test this hypothesis, we proposed to determine effects of infection on the behaviours that allow the insect to escape from the old cuticle. We used *R. prolixus* and *T. rangeli* as our model system. We expected to find effects of infection on ecdysis behaviour. We quantified mortality in infected insects and show that the high mortality during moulting in infected insects is probably influenced by the increased time spent to complete ecdysis.

In Chapter Three, we present an empirical study in which we hypothesize that mouting-related mortality under infection is increased because of the pathogen's impacts on its behaviour during ecdysis and/or alterations in levels of ecdysone. We also hypothesize that the delayed insect moulting process is related to delayed cuticle development (which in turn relates to the low glycogen available for biological energy spending) and impacts on nutrient absorption by the gut, as results of the microorganism infection. To test these hypotheses, we determined effects of infection on (i) cuticle thickness and development, (ii) levels of ecdysone, (iii) storage of glycogen in fat body, and (iv) morphology of midgut and hindgut epithelium. Here we also used *R. prolixus* and *T. rangeli* as our model system. We expected to find effects of infection on cuticle formation and ecdysone levels, as well as on glycogen content and midgut and/or hindgut morphology. We found that cuticle of infected insects takes a long time to be deposited, causing a prolonged time to moult and that may be related to the amount of glycogen in the fat body. Infected insects seem not to have damage in the midgut and hindgut epithelium.

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CHAPTER I

IMPACTS OF PARASITES ON INSECT MOULTING: A REVIEW

Article 1: Impacts of parasites on insect moulting: a review

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Abstract

Moulting is a crucial process for insect survival, as a new cuticle is produced and the old one is discarded. When an insect is parasitized, its physiology – including that related to moulting, can be impacted and, hence, insect growth can be altered. However, how different parasites can impact insect moulting is unknown; it would be interesting to verify a possible pattern in infections by different groups of parasites. We conducted a systematic review of impacts of parasites on insect moulting. We consider interactions between insects and: (i) protozoan parasites; (ii) a hypocrealean entomopathogenic fungus; (iii) a single-stranded RNA virus; (iv) baculovirus; and (v) parasitoid wasps. We show that insect moulting is impacted somehow by these parasites, the impacts being: mortality increases during moulting, moulting delays, non-moult and defects after moulting. We discuss some mechanistic aspects of the interaction between parasites and insect moulting and discuss how negative effects of parasites on insect moulting could be adaptive for parasites. The effects that parasites cause on insect moulting can be of interest in determining the outcomes of infections and can also help identify patterns of selection in the host and parasite.

Keywords: behaviour, ecdysis, infection, moult, parasitoid, pathogens, *Rhodnius prolixus*, survival, triatomine, *Trypanosoma.*

1. Introduction

Many studies have focused on understanding how parasites may affect insect survival and reproduction, but less attention has been given to insect development, especially moulting. Insect infections by parasites are based on a succession of steps in which host and parasite interact in specific ways. In the gut, protozoa and viruses have to overcome the insect defense as the gut cells secrete many enzymes and are immunologically very active (Daffre et al., 1994; Shanbhag and Tripathi, 2009). In contrast, entomopathogenic fungi have to penetrate the insect cuticle, which composes the first line of insect host defence for fungal infection (Chapman, 1998). And, on the other hand, parasitic wasps also have to overcome insect immune defense (Bianco and Maizels, 1989). To grow, insects need to shed their old cuticle (this specific step is called ecdysis), which occurs when changing from instar to instar. Before ecdysis, pathogen can affect insect by changes in nutrient content, which are necessary for insect development (e.g., Ferreira et al., 2010). During ecdysis, pathogen invasions can be facilitated because of insect vulnerability (Gullan and Cranston, 2014). In this case, mortality can increase and moulting defects occur, while moulting can be delayed or ecdysis may simply not occur (Grewal 1957; Tobie 1970; Watkins, 1971; Elliot et al., 2002, 2015; Rodrigues et al. 2016).

Some studies on insect infection have shown that insect moulting is impacted somehow by a pathogenic microorganism (Grewal 1957; Tobie 1970; Watkins, 1971; Elliot et al., 2002, 2015; Rodrigues et al. 2016). Such impacts may include mortality increases during moulting, moulting delays, non-moult and defects after moulting. We discuss these in more detail in the following sections. It is salient to note that, while these effects have been observed in three different systems, some authors are common to some of the different studies, so there may have been more interest in observing these effects by some groups than by others. However, how different pathogenic microorganisms can impact insect moulting and if such impacts could be a pattern found in infections by different groups of pathogens are interesting. It may be that these effects have not been noticed in other systems or by other authors, or not reported. Nevertheless, they may give insights into the physiology of infection in these systems, may be important in determining the outcomes of infections and may also help to determine patterns of selection on both host and pathogen. To answer the questions of how different pathogenic microorganisms can impact insect moulting, and if such impacts could be a pattern found in infections by different groups of pathogens, we conducted a systematic review. For this, we review studies on insect infection and its effect on the insect moulting process. For that we

consider interactions between insects and (i) kinetoplastida protozoans (Trypanosomatidae); (ii) a hypocrealean entomopathogenic fungus (Ascomycetes: Hypocreales); (iii) a single-stranded RNA virus; and (iv) parasitoid wasps. We describe these interactions in the contexts of moult and survival of insect hosts. Then, we discuss the mechanistic aspects of this interaction. Finally, we suggest how these negative effects could be adaptive for pathogens and/or insects and offer suggestions for future study. Discussing and studying effects of microorganism infection on insect moulting can deepen our understanding on the insect-pathogen interaction.

1.1. Hormonal regulation of insect moulting

In this section, we describe hormonal regulation of insect moulting according to Klowden (2013). In normal conditions, an insect moulting starts with epidermal cell retraction from the inner surface of the old cuticle, usually in an anteroposterior direction. Apolysis – which is the separation of the cuticle from the epidermis – is the next step. The subcuticular space formed after apolysis becomes filled with a secreted but inactive moulting fluid. Enzymes in the moulting fluid will be activated once the epidermal cells secrete the protective outer layer of the new cuticle. Then, while the new cuticle continues to be laid down, the inner part of the old cuticle (the endocuticle) is lysed and reabsorbed by the enzymes. Moulting begins when remnants of the old cuticle break down along the dorsal midline as a result of increased hemolymph pressure. In ecdysis, the insect breaks down its old cuticle along the dorsal midline and expands new cuticle both by swallowing air or water and/or increasing hemolymph pressure in different parts of the body. Thereafter, the body surface becomes sclerotized to form the exocuticle, and thus the insect completes the moulting process. We emphasize that, in insect larvae and caterpillars, most of the body cuticle remains membranous and the exocuticle is restricted to the cephalic region.

Hormones regulate the events mentioned above, and they stimulate the division of epidermal cells, which causes changes in the cuticle. Such hormones also stimulate the nervous system, which coordinates insect behaviors that are associated with moulting. The following three main types of hormones regulate insect moulting: (1) neuropeptides - including prothoracicotropic hormone (PTTH), ecdysis-triggering hormones (PETH, ETH), and hatching hormone (EH), (2) Ecdysteroids – e.g., ecdysone, and (3) Juvenile hormone (JH). Neurosecretory cells in the brain secrete PTTH. The PTTH initiates each moult by stimulating the synthesis and secretion of ecdysteroids by the prothoracic glands. The release of ecdysteroids then initiates

the changes in the epidermal cells that lead to the production of the new cuticle. Moulting characteristics are regulated by JH from corpora allata: JH inhibits the expression of adult characteristics – a high level of JH in the hemolymph is associated with moulting between two larval/nymphal instars, and a lower level of JH is associated to a moult between larva and pupa; JH is absent in the moult between the last nymph/pupa and adult stage. Specifically, moulting is regulated by the hormones ETH and EH. EH acts on the central nervous system, stimulating coordinated motor activities associated with the insect's exit from the old cuticle. PETH initiates pre-ecdysis behavior that loosens old cuticle muscle attachments, and ETH stimulates the release of EH from the brain. The neuropeptide bursicon controls exocuticle sclerotization and endocuticle deposition after ecdysis.

2. Methods

2.1. Systematic review

2.1.1. Eligibility Criteria and Information Sources

We performed a review of the literature by following the protocol of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses – PRISMA, 2020 (Figure S1) (Page, 2020). For this, we searched for information that indicates the study is related to moulting of diseased insects, and the eligibility criteria for inclusion of studies were based on papers that present records of interaction between insects and (i) protozoans, (ii) entomopathogenic fungi, (iii) entomopathogenic virus, or (iv) parasitoids We conducted the literature searches from July to October 2023, in the Web of Science–Core Collection database. The searches were performed without limitation of date.

2.1.2. Strategy of search, selection of studies, and collection of data

We performed the searches in each database using each keywords separately, for the following terms: ‘insect AND moult* AND infect*’; ‘insect AND moult* AND infect*’; ‘insect AND moult* AND protozoan; ‘insect AND molt* AND protozoan’; ‘insect AND moult* AND parasite*; ‘insect AND molt* AND parasite*’; ‘insect AND moult* AND pathogen*; ‘insect AND molt* AND pathogen*’; ‘insect AND moult* AND entomopathogen*; ‘insect AND molt*

AND entomopathogen*'; 'insect AND moult* AND fung*; 'insect AND molt* AND fung*'; 'insect AND moult* AND virus*; 'insect AND molt* AND virus*'; 'insect AND ecdysis AND infect*' OR 'insect AND ecdysis AND protozoan' OR 'insect AND ecdysis AND parasite*' OR 'insect AND ecdysis AND pathogen*' OR 'insect AND ecdysis AND entomopathogen*' OR 'insect AND ecdysis AND fung*' OR 'insect AND ecdysis AND *'. For each record identified through database searching, we evaluated the title, abstract, and keywords. For studies that provided information regarding effects of pathogenic microrganisms on insect moulting, we searched the full text, looking for such effects. After applying our inclusion criteria, a total of 21 studies were included from the search on databases.

2.1.3. Items of data

We divided the data into four main sets to perform the analyses: effects on moulting, and host and pathogenic microorganism diversities. The description of effects on moulting were based on results reported by the studies. Categorical variables consisted of effects such as defective moult, mortality, time to moult, and successful moult.

2.1.4. Systematic review approach

The database search identified 70 records for the 16 keywords, which were then filtered to 60 records after removing the records in which the main subjects were not related to effects of pathogenic microrganisms on insect moult. We screened 60 papers from the database. After duplication removal (n = 10), we removed two studies that did not provide access to the full text (n = 2). Among the remaining full-text articles (n = 48), 22 studies were excluded (i) for not providing effects of parasites on insect moulting description or showing inaccurate records, and (ii) for providing repetitive information or not primary research (n = 5). The analyses were then performed with 21 selected articles (Figure S1).

3. Results and Discussion

3.1. Overview of parasite effects on insect moulting

Here we describe effects of parasites on insect moulting. Most of the studies that consider the effects of a pathogenic microorganism infection on insect moulting are related to triatomines and some of these studies have SLE as an author. These insects are indeed a model system for insect physiology and especially the process of moult (which was first investigated by Sir Vicent B. Wigglesworth) as this can be controlled in these insects by offering blood meals.

In triatomine hosts, moulting effects have been shown from infection by two trypanosome parasites, *Trypanosoma cruzi*, that is vectored by triatomines and causes Chagas disease in humans, and *T. rangeli*, that is more typically considered a parasite of the triatomines (*Rhodnius* spp.) themselves and is used as a model to study triatomine-Trypanosoma interactions. Two studies have demonstrated effects of *T. cruzi* (Trypanosomatidae) on triatomine moulting (Botto-Mahan, 2009; Elliot et al., 2015), including increased mortality and delayed moult (Table S1). In contrast, many others report effects of *T. rangeli* (Trypanosomatidae) on moulting (Brecher and Wigglesworth, 1944; Watkins, 1971; Grewal, 1957; Tobie, 1970; Ferreira et al., 2010; Rodrigues, et al., 2016) (Table S1). These probably occur because of the knowledge about *T. rangeli* affecting insect biology like increased mortality (e.g., Rodrigues et al. 2016), reduced fecundity and fertility (e.g., Fellet et al., 2014) and negative phototaxis (e.g., Marliére, et al., 2015). In relation to the effects *T. rangeli* causes on *Rhodnius* sp. (Hemiptera, Reduviidae: Triatominae) (as *T. rangeli* can only infect *Rhodnius* species), there is an increased mortality during insect moulting and a prolonged moulting period, which was also seen for *T. cruzi* (Botto-Mahan, 2009; Elliot et al., 2015) (Table S1). In addition to that, we have increases in moulting defects as well as increases in absence of moult in *T. rangeli*-infected insects (Brecher and Wigglesworth, 1944; Watkins, 1971; Grewal, 1957; Tobie, 1970; Ferreira et al., 2010; Rodrigues, et al., 2016) (Table S1).

We are aware of one example of fungal infection affecting insect moult in the literature, which is on locusts being infected with *Metarhizium anisopliae*. Here, infection delays moult and increases insect mortality, besides being able to thermoregulate (and so delay death) (Elliot et al., 2002).

The single-stranded RNA virus, *Triatoma virus* (TrV) is shown to delay and inhibit *Triatoma* moulting (Muscio et al., 1997), besides impairing oogenesis (e.g., Rozas-Dennis and Cazzaniga, 2000), similar to what we have seen for parasites and fungi insect infections. However, it is not reported for TrV insect infection if mortality is increased during moulting (even though overall mortality is increased) (Cardoso et al. 2022). Interestingly, in a coinfection, where triatomines are previously infected with TrV and secondly infected with *T. cruzi*, in 60 days all nymphs could moult to 4th instar (Marti et al., 2017). However, when insects are previously infected with *T. cruzi*, and with TrV secondly, nymphs moulted to 4th instar within 35 days. When the infection occurs simultaneously, no reference to moult is given in the paper, but infection is similar between both microorganisms (Marti et al., 2017).

During baculovirus infection, enzymes can inactivate the insect moulting hormone (20-hydroxyecdysone) due to an addition of the group uridine diphosphate-sugar (USD-sugar), what inhibits moulting (O'Reilly and Miller, 1991). Second instar *H. exigua* parasitized by *Trichoplusia ni* moulted to the 3rd instar perfectly, but the length of the 3rd instar was 24h longer than in control, and their development was blocked after moulting to the 4th instar (Thompson, 1982). Interestingly, mortality was associated with moulting as infected insects (*Lacanobia oleracea* injected with metalloproteinases of venom glands of the ectoparasitic wasp *Eulophus pennicornis*) death occurred right before or during its ecdysis, with most of the insects failing to complete moult (Price et. al, 2009).

The principal effects caused by infection are increased mortality, delayed moult period, absence of moult and defective moult. Such effects are reported for groups of Hemiptera (i.e., triatomines) and Orthoptera, which are hemimetabolous insects. Meanwhile, one study has examined this in locusts (Orthoptera) infected by fungi. Before describing each in detail, we give here a brief overview of each of the four study systems in which moulting has been shown to be affected by infection.

3.2. Effects of *Trypanosoma cruzi* and *Trypanosoma rangeli* on triatomine moulting

(i) Triatomines

Triatomines are hematophagous insects of the family Reduviidae, subfamily Triatominae. To complete development, the five nymphal stages and the adult stage need to feed on blood. These hematophagous insects colonize houses, emerging at night from cracks in the walls, for example, to feed on the people who live there (Dias, 1987). Many species of these blood-sucking insects can act as vectors of Chagas disease (Dias, 2016), and they can transmit *Trypanosoma cruzi* during their entire life cycle when infected (Hoare 1972). However, the triatomines *Triatoma infestans* (Klug, 1834), *Rhodnius prolixus* (Stål, 1859) and *Panstrongylus megistus* (Burmeister, 1835) are considered the most efficient, and thus relevant, triatomine vectors of Chagas disease (Garcia and Azambuja, 1991). Triatomines may ingest the parasites from humans by a bite, but these insects can also be infected with trypanosomes from reservoir animals. Besides being vectors of *T. cruzi*, triatomines can act as vectors of other trypanosomatids such as *T. rangeli* and *Blastocrithidia triatominae*. These are also of interest due to their pathogenicity in the insect vector (Schaub and Breger, 1988), which includes effects on moulting and and/or survival. *Trypanosoma rangeli* is a good microorganism to be used as a model in many studies as it infects the insect similarly to other insect pathogenic microorganisms that cross a barrier (in this case, the intestinal epithelium) and infect the haemolymph.

(ii) Triatomine infection by *Trypanosoma cruzi* or *Trypanosoma rangeli*

The life cycle of *T. cruzi* has several developmental stages in insect vectors and mammalian hosts. *Trypanosoma cruzi* has three main forms: (i) amastigote, (ii) epimastigote, and (iii) trypomastigote. The amastigote is a proliferative form and is found inside vertebrate host cells; the epimastigote is also a proliferative form, but is found in the intestine of insect hosts; and, finally, the trypomastigote is the infective form found in vertebrates and insect hosts (Rassi et al., 2010). The triatomine ingests trypomastigote forms during the blood meal in infected mammals. A few hours after infection, trypomastigotes migrate from the anterior midgut to the posterior midgut where they differentiate into epimastigote forms. Later, they differentiate into their infective form and are eventually released together with insect feces. For

the infection of an insect by *T. rangeli*, a parasite that also has the three main forms, the epimastigote forms need to eventually cross the intestinal epithelium, reaching the haemolymph, where these microorganisms multiply and invade insect salivary glands. In these glands, the microorganisms transform into the infective forms, which can be transmitted to a vertebrate in the saliva of the insect when feeding. For more details about *T. cruzi* and *T. rangeli* development and differentiation, see Guarneri and Lorenzo (2017, 2021).

(iii) Triatomines × *Trypanosoma*

According to the previous information, triatomines accommodate trypanosomal parasites in a complex network of interactions that range from insects to vertebrate process of transmission, that goes from the insect to a vertebrate host (Garcia and Azambuja, 1991). From that, these parasites developed exploitation mechanisms, that ultimately improve their transmission. For instance, parasites produce specific molecules that alter insect physiology and so behaviour (Herbison et al., 2018). Infection by *Trypanosoma cruzi* leads to behavioural and/or physiological changes that could be increases in feeding, biting frequency or shorter defecation time (Botto-mahan et al., 2006; Marliére et al., 2015; Guarneri and Lorenzo, 2017).

Since some parasites have long been considered avirulent to their insect vectors, only a few studies have investigated how *T. cruzi* might impact triatomine biology. (Marliére et al., 2015; Fellet et al., 2014). For instance, *T. cruzi* (Botto-Mahan, 2009; Elliot et al., 2015) and *T. rangeli* (Brecher and Wigglesworth, 1944; Watkins, 1971; Grewal 1957; Tobie, 1970, Ferreira, et al., 2010; Rodrigues, et al., 2016) are shown to cause high mortality during moulting of *R. prolixus* and also delay insect moult in the survivors. For *T. rangeli*, insect moult defects and absence of moult are also reported (Ferreira et al., 2010; Rodrigues et al., 2016). The delay in moulting could possibly prolong the time taken to reach the adult life stage. We expect that such a delay would affect insect fitness. *Trypanosoma cruzi* takes approximately up to a month at 28°C to develop into infective stages (Schaub, 1989). As the insect will feed again after they have moulted, the delayed development of the insect could well favor the parasite if insect moult, and thus the next blood-meal, were delayed until a time that the parasite is in the form to be transmitted to a vertebrate host. Therefore, the delay could be expected to benefit parasite transmission, as it has time to complete its development inside the host before the next blood feeding.

3.3. Effects of entomopathogenic fungi on insect moulting

(i) The entomopathogenic fungi

Many fungal species have been documented to infect insects (Hajek, 1997; Hajek and Delalibera, 2010). Highly specialized pathogens of arthropods are to be found within the phylum Zygomycota (specifically within the subphylum Entomopthorales). However, the best known, and perhaps most widespread entomopathogenic fungi are found in the order Hypocreales, phylum Ascomycota. The best studied, especially for biological control, are the genera *Beauveria* and *Metarhizium*, and most of the studies that are related to microbial control of insects with fungi consider these genera. Unlike many other microbial pathogens which need to be ingested to infect their hosts (e.g., viruses and bacteria), entomopathogenic fungi infect their hosts via penetration of the cuticle.

(ii) Insect infection by entomopathogenic fungi

Infection by an entomopathogenic fungus starts after its contact with the insect cuticle. That is the first barrier to be beaten by the fungus before initiating the insect infection process. The process of infection by entomopathogenic fungi occurs mainly through the cuticle. Entomopathogenic fungi are able to degrade the insect host cuticle hydrocarbons via enzymatic degradation (Napolitano and Juárez, 1997). The infection process can be divided into three stages: (i) adherence of spores on the cuticle of the host insect, germination is activated by carbohydrates present in the cuticle, development of appressorium and penetration peg formation; (ii) cuticle penetration, which is the result of combined action of mechanical force of a penetration peg and the action of enzymes that the fungus has secreted; such enzymes break down the cuticle, and the fungus can obtain nutrients; and (iii) fungus proliferation in the insect hemocoel with the formation of blastospores (Hajek and St. Leger, 1994). Thus, the insect body is colonized by the fungus leading to insect host death. The fungus grows in the host, under suitable conditions, and external conidia are produced on the dead host (Hajek and St. Leger, 1994; Goettel et al., 2010), which facilitates the horizontal transmission of the fungus.

(iii) Locusts × *Metharhizium*

In addition to causing insect mortality, and being used in the biological control of insects, entomopathogenic fungi, as *Metarhizium*, can promote effects on locust biology by impairing moulting. Nymphs of the orthopteran *Schistocerca gregaria* that were infected with *Metarhizium* and were able to thermoregulate (and so delay death) had delays in moulting of a few days when compared with controls (Elliot et al., 2002). It is interesting to say that the fungus will only benefit in an infection if insect spends more time to moult, and so the fungus can complete its infection through the insect cuticle before ecdysis (but see Duneau and Ebert, 2012).

3.4. Effect of virus on insect moulting

(i) The *Triatoma virus* (TrV)

The range of viral species that can infect triatomine insects is almost unknown. To date, eight positive single-strand RNA viruses, namely *Triatoma virus* (TrV) (Picornavirales, Dicistroviridae: cripavirus) and *Rhodnius prolixus viruses* 1 - 7 have been investigated in more detail, but only the *Triatoma virus* has been shown some effects on insect moulting. The *Triatoma virus* is a spherical non-enveloped virus found in *Triatoma infestans* (Muscio et al., 1987, 1988). The viral genome is a positive-sense single-stranded RNA molecule (Czibener, et al., 2000).

(ii) *Triatoma* infection by *Triatoma virus* (TrV)

The *Triatoma virus* replicates within the cytoplasm of cells of the digestive tract of triatomines (Muscio et al., 1988). The initial infection occurs when a susceptible host insect feeds on triatomine cadavers, for example, that are contaminated with the occluded form of the virus. Entry of the viral genome into the cell begins with the viral particle binding to a specific receptor on the outside of the cell. Once bound to a receptor, the RNA genome into is released into the cell (Flint, 2015). Viral transcription and replication occur in the cell nucleus and new virus particles are budded out from the basolateral side to spread the infection systemically (Flint, 2015).

(iii) *Triatoma* × *Triatoma virus* (TrV)

Individuals infected with the *Triatoma virus* (TrV) show physiological changes, such as moulting failure or leg paralysis (Muscio et al., 1987). Also, infections by TrV were shown to induce 97.6% of overall mortality in blood-fed fifth instar nymphs and to delay the moulting process increasing development time (Muscio et al., 1997; Rozas-Dennis and Cazzaniga, 2000; Vieira et al., 2018) and, additionally, impair oogenesis (Rozas-Dennis and Cazzaniga, 2000). A much smaller proportion of the progeny of the females from infected stock than of the uninfected females successfully moulted to second-instar nymphs (44% *v*. 80%) (Muscio, et al., 1997). The progeny of the females from the infected stock developed relatively slowly, spending around 6.1 days as first instars - compared with 3.9 days for the progeny of uninfected females (Rozas-Dennis and Cazzaniga, 2000). Furthermore, TrV appears to inhibit the moulting process. For instance, absence of moult in *Triatoma infestans* was also higher than controls (3^{rd} instar nymphs: Control=100% moulted, Infected=20.3% moulted; 4^{th} instar nymphs: Control=94% moulted, infected=10.8% moulted; 5^{th} instar nymphs: Control=85% moulted, infected=3.1% moulted) (Muscio, et al., 1997). Moreover, for insects previously infected with TrV and that were infected with *T. cruzi*, all nymphs moulted to 4^{th} instar nymph stage (Marti, et al., 2017). Based on that, we speculate if there is any virus in other triatomine species that impact insect moult in a stronger way that increases the effects found on moulting.

iv. Caterpillars × Baculovirus

Baculoviruses can manipulate their insect hosts in a variety of ways to facilitate their propagation. A gene that has been extensively studied in this group is the *egt*, and many baculoviruses possess an *egt* gene (Cory et al., 2004). The *egt* is secreted from infected cells into the hemolymph of infected larvae, where it catalyzes ecdysteroid conjugations. This blocks the ecdysteroids, the insect moulting hormones and keeps the host in the larval stage (O'Reilly, et al., 1989). It's important to note that infected insects can present low levels of ecdysteroid (O'Reilly, et al., 1989).

3.5. Caterpillar × Parasitoids

Parasitoids have been shown effects on insect development, what includes moulting. For instance, the caterpillar *Trichoplusia ni* is parasitized by the parasitoid wasp *Hyposoter exiguae*, caterpillar development is delayed and the authors argue that this may be related to the degradation of juvenile hormone by the activity of juvenile hormone esterase (Thompson, 1982). The regulation of the host's development and the prevention of the following stages can be a suitable environment for parasite development.

3.6. Mechanistic aspects of the interaction between insect moulting and parasites

Here we present possible explanations for the main effects (i.e., high mortality during moulting, delayed moulting, absence of moult and defective moult) that parasites can cause in insect moulting.

The delayed moult as well as the absence of moult could be indirectly related to nutrient availability. We also showed that cuticle thickness is related to moulting delay, and that such thickness seems to be related to the amount of glycogen in the fat body of the insect (see Chapter II). For instance, lipids and fat body were increased in insects infected with *T. rangeli* (Ferreira et al. 2010; AAG, unpublished data). It is proposed that this is due to parasite sequestration of lipids from the ingested blood by its host. As is known, *Trypanosoma rangeli* uptakes lipids from the haemolymph of *R. prolixus* and internalizes them through a specific receptor on the parasite external surface (Folly et al. 2003).

The increased mortality could be related to the deformities that impairs normal insect development, leading to death. Interestingly, insects that died during moulting became infected with bacteria, and the fungus did not do well (Elliot et al., 2002). The time spend to finish ecdysis possibly impacts insect survival, since the new cuticle could start sclerotizing before finishing ecdysis, with incomplete ecdysis and insect death. Thus, insect death during moulting may be related to failure to successfully complete ecdysis, ecdysone low levels, and/or also moulting defects. In Chapter II, we have quantified the moulting-related mortality in diseased insects, and we suggest insect mortality is influenced by the time taken to complete ecdysis and ecdysone levels. Ecdysone levels are also an important component of insect survival, since it is a molecule that has a role in the moulting process but also in the immune system (Azambuja et al., 2017).

We demonstrated that parasitized insects have low levels of ecdysone (Chapter II), which possibly impacts the beginning of the moulting and could also impact insect survival. Would this outcome be behavioural manipulation or host defence? We suggest that the changes in ecdysone levels are the result of a manipulation of the host by the parasite, because the ecdysone levels found are low and, as we said before, ecdysone also participates in the immune system. Thus, it is plausible to think that if the low levels of ecdysone found were an insect defence, then ecdysone levels should be high rather than low.

3.6.1. Interaction between parasite and symbiont and its effects on insect moulting

The delayed moult and absence of moult could also be impacted by indirect effects of the pathogen to a host symbiont, which improves insect nutrition. Such effects could indirectly interfere with insect development. For instance, the bacterium *Nocardia rhodnii*, found in the esophagus and midgut of *R. prolixus*, is essential for the insect's normal growth (Harington, 1960). These symbionts provide folic acids, pyridoxine, thiamine, nicotinic acid and riboflavin to the insect; these nutrients can be used for normal development and ecdysis (Lake and Friend, 1968). Moreover, when the blood-sucking bug, *R. prolixus* is infected with the protozoan *T. rangeli*, the parasite inhibits *N. rhodnii*, resulting in a vitamin deficiency that can lead to metabolic defects, and thus increase moulting defects, absence of moult, and increase mortality (Watkins, 1971; Rodrigues et al., 2016). It has been suggested that the amino acid tyrosine (Tyr) produced in significant quantities by symbiotic bacteria during insect development is also involved in insect cuticle formation (Anbutsua et al., 2017). Thus, if an insect is infected with a parasite that impacts this symbiont, it can, consequently, interfere with nutrient availability for the insect development. However, there is the possibility of competition between the insect host and the parasite for nutrients, that we expect to be important for cuticle formation (Tobie, 1970; Xia, Clarkson and Charnley, 2002). Also, it has been shown that the triatomine *R. prolixus* infected with *T. rangeli* were unable to moult while others died (Grewal, 1957), and possible mechanisms explaining deformation and absence of moult as well as moulting delay in infected insects may be related to vitamin deficiencies (Watkins 1971; Grewal 1957; Tobie 1970). Such mechanisms need to be studied in more detail, as we usually find this information only in a descriptive way in literature and, apparently, not so much attention is given to that.

3.7. Adaptive aspects of the interaction between insect moulting and parasites

The delayed and defective moult could be adaptive for the parasite. In the case of *Trypanosoma*, delays in moult cause a delay in the next blood-meal taken and may thus allow the parasite to complete its development inside the host insect and be transmitted to a possible vertebrate host. The same thing can happen for the caterpillar *Trichoplusia ni* that is parasitized by the parasitoid wasp *Hyposoter exiguae* – the caterpillar development is delayed and so, the parasitic wasp can complete its development inside the host. Moreover, defective moult could also be related, ultimately, to pathogen transmission. For example, defects caused by *Trypanosoma* can debilitate the insect, what could increase insect vulnerability to predation. In the case of entomopathogenic fungi, if the defect causes inability to walk or fly meaning that the insect can fall and so get in contact with the soil – where fungi can be found – this microorganism may be better to sporulate and infect other insect hosts.

It is harder to imagine how delayed moulting might be adaptive for the hosts, although the possibility cannot be discarded without due consideration. One possibility, in the case of triatomines infected with *Trypanosoma*, is that if the insect does not have a vertebrate to feed after ecdysis, it is good not to shed the old cuticle, once the insect can die because of food starvation - since soon after ecdysis it needs to blood-feed.

3.8. Directions for future research

The principal effects of infection in insects are increased mortality, delayed moult period, absence of moult and defective moult. Such effects are reported for groups of Hemiptera (i.e., triatomines) and Orthoptera, hemimetabolous insects. While it would be interesting to determine if this occurs in other hemimetabolous insects, it is perhaps more important to determine if it occurs in holometabolous insects. However, it would be difficult to observe moulting effects in this group. They have a different pattern of development, which involves the stage of pupae. But there could also be a delay in moulting, because of nutrients sequestration by the pathogen.

Related to moulting delay and/or defective moults, studies on amino acids or proteins content (which are important for cuticle formation) in haemolymph would be an interesting point to study, as we said, pathogens can compete for nutrients of its host and amino acids could

be one of them. We suggest to do comparations of the number of proteins the insect has when infected. That could be one component of the mechanisms behind the moulting delayed.

We suspect that some insects die because they have problems in air swallowing when shedding their old cuticle. This air is used to inflate the body and so shed the old cuticle. As an example, infection of *R. prolixus* with *T. rangeli* may cause tracheal damage (Watkins, 1971), but it is not well demonstrated due to lack of technology at the time. Thus, we suggest studies of the histology of the tracheal system of infected insects.

In view of the hormonal issue, the edysone level is an important variable to be measured under a pathogen infection, since this hormone can play a role in improving insect immunity besides triggering moulting (Azambuja et al., 2017). So, we suggest studies measuring ecdysone levels to see how they fluctuate in pathogen-infected insects, considering that it can act both in immunity and in moulting.

4. Conclusion and remarks

In this review, we addressed the question of what is the current state of our understanding of microorganism effect on insect moult and if such effects could be a pattern found in infections by different groups of microorganisms. We showed that there are negative impacts on the insect moulting process, such as moulting delay, absence of moult and defective moult, besides increased mortality during moult. Such effects seem to be conserved among the following distinct groups of microorganisms, protozoan, fungi and virus, when infection occurs. However, the mechanisms behind these have not been investigated, having only speculations about that. Possible explanations can be related to nutrient availability (Tobie, 1970; Xia, Clarkson and Charnley, 2002), cuticle damage, behaviour during ecdysis and/or ecdysone levels (see Chapter II of this thesis). Moreover, delaying moult seems to be adaptive for the pathogen, because it delays the insect's next blood-meal (that will occur only once the insect has moulted). Thus, it would allow the parasites time to develop, favoring parasite transmission. Moreover, pathogens can weaken the process of insect moult physiology in a way that pathogen emergence is facilitated (as with entomopathogenic fungal that must pass through the cuticle to emerge and sporulate). Therefore, studies involving possible mechanisms that explain mortality during moult, moulting delay, absence of moult and defective moult during an infection would be interesting. We would like to highlight that many studies do not report effects of

entomopathogen infections on insect moulting, which would be crucial for our deep understanding of insect moult physiology under pathogen infections.

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Supplementary material

Table S1. Some examples of effects of different parasites on insect moulting.

Insect host	Insect-host order	Parasite	Effects of the infection on moulting	Reference
Locusts	Orthoptera	*Metarhizium anisopliae*		Elliot et. al, 2002
Blood-sucking bug (*Rhodnius prolixus*)	Hemiptera	*Trypanosoma rangeli*	Delayed moult; Increased mortality	Fellet et al. 2014
Blood-sucking bug (*Rhodnius prolixus*)	Hemiptera	*Trypanosoma cruzi*	Delayed moult; Increased mortality; Moult defects; Inhibited moult	Elliot et al. 2015; Botto-Mahan et. al; Watkins, 1971; Grewal, 1957; Tobie, 1970; Rodrigues et al. 2016; Brecher and Wigglesworth, 1944; Ferreira et. al 2010.
Blood-sucking bug (*Triatoma infestans*)	Hemiptera	*Triatoma virus* (TrV)	Delayed moult and inhibit moult	Rozas-Dennis and Muscis et. al, 1997
Caterpillar	Lepidoptera	*Baculovirus*	Delayed moult	O'Reilly, et al., 1989
Caterpillar (*Trichoplusia ni*)	Lepidoptera	Parasitoid wasp (*Hyposoter exiguae)*	Delayed moult	Thompson, 1982

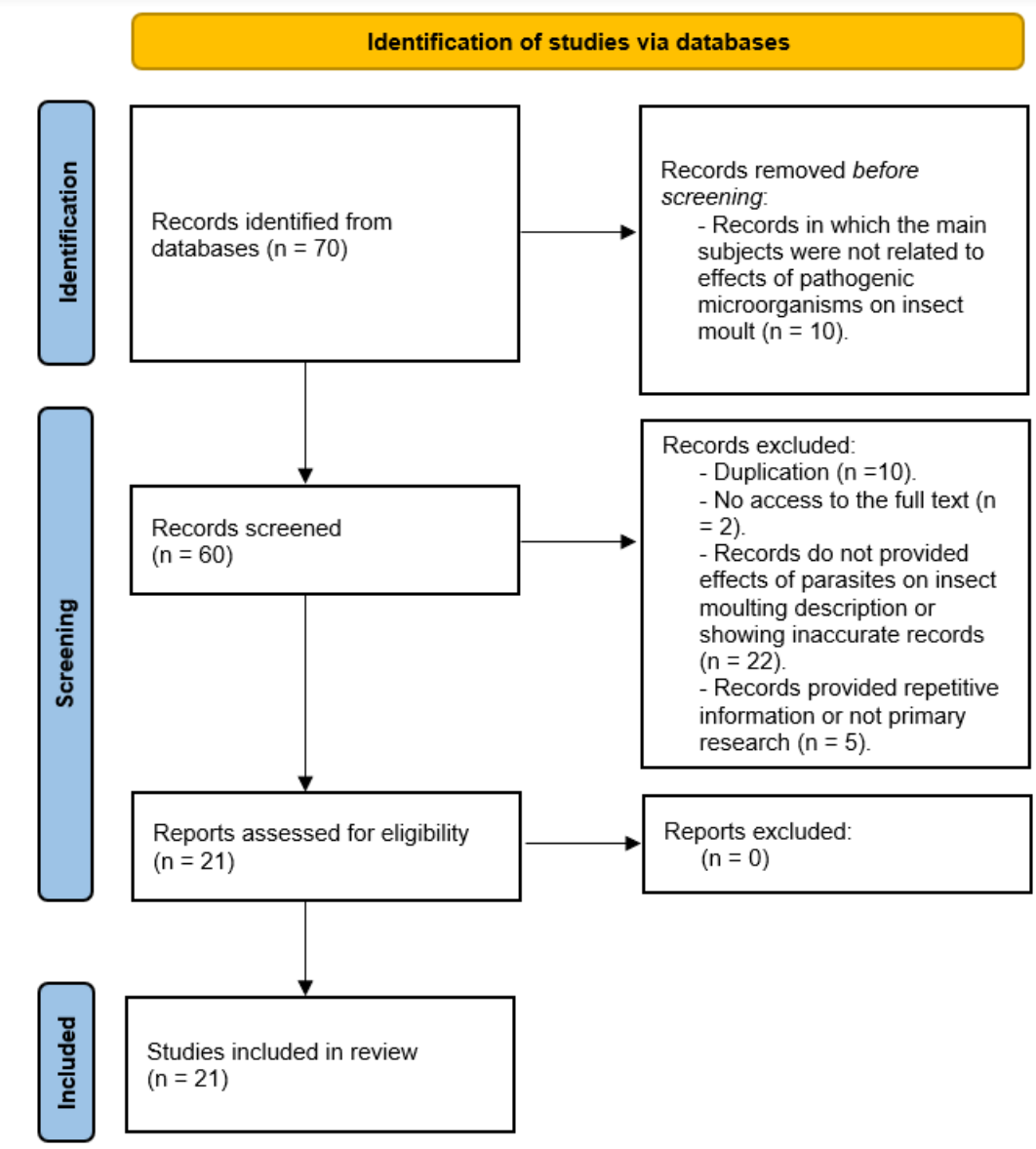

Figure S1. Global view of the records through database searching. This diagram shows the main results of the searches for papers that presented effects of pathogenic microorganisms on insect moulting. We conducted the literature searches from July to October 2023 at the database Web of Science – Core collection (Page et. al 2020).

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CHAPTER II

DECREASED SURVIVAL OF PARASITE-INFECTED INSECTS IS ASSOCIATED WITH INCREASES IN THE TIME REQUIRED TO COMPLETE ECDYSIS

Article 2: Decreased survival of parasite-infected insects is associated with increases in the time required to complete ecdysis

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Abstract

Insect development depends on the success of moulting, which is the process of producing a new cuticle and shedding the old one. While there is evidence that insects infected by parasites can experience increased mortality during moulting, this phenomenon is not well quantified or understood. Here we used the triatomine bug *Rhodnius prolixus* (Hemiptera: Reduviidae) and the protozoan parasite *Trypanosoma rangeli* as a model system to address this. We measured survival during moulting of insects under infection. Mortality of 4th instar *T. rangeli*-infected nymphs was 6% higher than that of uninfected insects. Infected insects showed more defective moult as well as absence of moulting. Moulting was initiated at 42 days after the blood meal in infected insects versus 17 days in uninfected insects, showing a delay in moulting. We also analyzed ecdysis-related behaviour of insects under infection. Infected insects took longer to complete ecdysis, specifically at the beginning (*ecdysial line disruption*) and the end (*exuvium release*) of ecdysis than did uninfected insects. We show that the high mortality of infected insects is associated with greater time taken to complete ecdysis. Our results indicate likely effects of infection on the behaviours that allow the insect to escape from the old cuticle, and help understanding fundamental physiological processes of insect development under infection.

Keywords: Behaviour, ecdysis, moulting, pathogen, *Rhodnius prolixus*, survival, triatomine, *Trypanosoma.*

1. Introduction

To grow and develop, insects have to moult, that is the process in which a new cuticle is produced and replaces the old cuticle. Moult includes ecdysis, the shedding of the old cuticle at the end of the moulting process, although the term 'ecdysis' is often used imprecisely to denote the entire process. The behaviours characteristic of ecdysis are sequential and are critical for insect survival (Chapman, 2013). High mortality during moulting has been shown in triatomine bugs (Hemiptera) or grasshoppers (Orthoptera) when infected with protozoan, fungal or viral parasites (Brecher and Wigglesworth, 1944; Grewal, 1957; Tobie, 1970; Watkins, 1971; Elliot et al., 2002, 2015; Botto-Mahan, 2009; Ferreira et al., 2010; Rodrigues, et al., 2016; Muscio et al., 1997). To understand physiological processes of insect development under infection, as well to understand adaptive relationship between insect and parasites, it is necessary to obtain quantitative data on mortality during moulting of diseased insects, as well as to examine insect behaviour during ecdysis.

Behaviour is an important component to be considered in moulting, beyond hormonal regulation. Once the new cuticle has formed, insects are ready for ecdysis, the last step of the moulting process (Wigglesworth, 1934; Chapman, 2013). Here, the behaviours performed by the insect can be divided into distinct phases as has been done in crickets (Carlson, 1977), locusts (Hughes, 1980), and triatomines (Ampleford and Steel, 1981). Specific behaviours occur in a sequential manner, allowing the insect ultimately to release its old cuticle and adopt a position in which the new cuticle can harden. If a given behaviour cannot be completed adequately, the insect may be deformed in its new instar or be unable to escape its old cuticle (e.g., Ampleford and Steel, 1981). When an insect is infected by a parasite, its hormonal physiology can be affected and therefore its moult as well.

Since the first studies on insect physiology by Wigglesworth (1934), *R. prolixus* has been used as a model organism, in particular to study moulting. The insect is comparatively easy to maintain in laboratories and can survive months of starvation. It is also one of the best-known insect species in terms of its physiology (Guarneri and Lorenzo, 2021), making it an excellent model. Once fed to repletion, nymphs moult to the next instar, allowing experimental control of the timing of this process (Wigglesworth, 1934; Azambuja et al., 2017). Also, this insect is one of the most important vectors of Chagas disease (Garcia and Azambuja 1991). *Trypanosoma rangeli*, a parasite that infects *R. prolixus,* is also used as a model to study infection in triatomines. Unlike *T. cruzi*, it infects the insects in a similar fashion to other

pathogenic microoganisms of insects in that it must cross a barrier (here, the gut epithelium) in order to reach the haemolymph.

Here we document insect mortality during moulting so as to generate hypotheses regarding the underlying, focusing especially on the insect's behaviour. We examined *R. prolixus* infected by *T. rangeli*. To understand pathogen-induced mortality on insect moulting we quantified mortality during moulting of infected insects. These insects, when infected by *T. rangeli*, die more during moulting and they also present more defective moults compared to uninfected insects (e.g., Rodrigues et al., 2016). We hypothesized that the insect's behaviour during ecdysis is altered due to the infection and this alteration impacts moulting. To investigate this, we examined ecdysis in uninfected and infected nymphs of *R. prolixus*. We observed and recorded ecdysis-related behaviours of infected insects, followed by measurements of the time of each ecdysis-related behaviour that infected and uninfected insects performed. These qualitative and quantitative data differ from previous studies that present qualitative data alone (Carlson, 1977; Hughes, 1980; Ampleford and Steel, 1981). Quantitative data are important to confirm differences between treatments. By that, we propose to determine effects of infection on behaviours that may normally allow the insect to escape from the old cuticle.

2. Material and Methods

2.1. Experimental organisms

Rhodnius prolixus were obtained from a colony maintained in the laboratory at the René Rachou Institute (FIOCRUZ, Minas Gerais, Brazil). The colony was established from insects collected in Honduras in 1990 and maintained under laboratory conditions (26 ± 1°C; 65 ± 10% RH; and natural illumination cycle). As these insects are obligatory hematophages and need blood during all active phases of their life cycle, they were fed on mice that had been anesthetized with an intraperitoneal injection containing ketamine (150 mg/kg; Cristália, Brazil) plus xylazine (10 mg/kg; Bayer, Brazil). Triatomines are hemimetabolous insects whose development includes eggs, five nymphal instars and adults. Here we used 4th instar nymphs of *R. prolixus* for all experiments.

The CHOACHI strain of the parasite *T. rangeli* used in this study was isolated from naturally infected *R. prolixus* (Schottelius, 1987). These are the same strains of insect and parasite used in Rodrigues et al. (2016). When *R. prolixus* feeds on *T. rangeli*-infected

mammals, parasite development begins: the trypomastigote forms, ingested with the blood meal, reach the midgut and differentiate into epimastigotes, becoming able to colonize the entire intestinal tract and potentially cross the intestinal epithelium reaching the haemolymph; in the haemolymph, the parasites multiply and migrate to the salivary glands, where epimastigotes differentiate into metacyclic trypomastigote forms; this form will be transmitted to the vertebrate host through the next insect bite (Tobie, 1970; D'Alessandro, 1976; Hecker et al., 1990). To infect triatomines, we used the parasite epimastigote forms, cultured by weekly passages at 27°C in liver-infusion tryptose (LIT) medium, and supplemented with 100 units/ml of penicillin, and 100 mg/ml of streptomycin. To maintain the infectivity of the parasite strain, it was passed through triatomines and mice every 15 passages.

2.2. Experiment I: Survival analyses and time to moult

To measure insect survival, three days after parasite inoculation into the insects' hemocoel, groups of 4th instar nymphs were transferred to Petri dishes (60 × 15mm) and kept in a climate-controlled room (24 ± 1 °C; 60 ± 10% RH, and 12:12 L:D). This experiment was conducted in a randomized block design, with two treatments (control or infected) and four replicates per treatment. Each replicate had five insects (N=20 insects per treatment). Mortality rates were recorded daily up to 60 days after the first moult. We also recorded successful moults, non-occurrences of moults and defective moult (i.e., insects presenting morphological alterations after moulting, such as leg deformities; Watkins, 1971; Rodrigues et al., 2016). We adjusted a Linear Mixed Model (LMM), with normal distribution and random intercepts to evaluate the significance of explanatory factors: treatment and cumulative effect of infection (mortality, absence of moult, morphological defects, successful moult). The distribution was evaluated based on residuals of the model and we opted for the binomial adjustment. The explanatory variables consisted of: (i) absence of moult, (ii) morphological defects, (iii) successful moult (iv) and treatment. Percentage of insects was the response variable. The Petri dish was considered as a random factor in the analysis. We also analyzed insect mortality over time using Kaplan–Meier survival analyses. Comparisons between the two treatments were made with log-rank tests.

In addition to the above, to determine the effect of *T. rangeli* infection on the time required by 4th instar nymphs of *R. prolixus* to moult to 5th instar, we performed a Kaplan–Meier survival analysis with moulting as the event. Comparisons was made with log-rank tests.

Statistical analyses were conducted using the software R (R Core Team 2019).

2.3. Experiment II: Ecdysis-related behaviour

To obtain behavioural sequences of insects attempting to escape from the old cuticle, thirty 4th instar nymphs (fifteen from each treatment) were observed individually in Petri dishes (90mm × 15mm). On day 14 after feeding, fifteen 4th instar nymphs from the control and fifteen infected nymphs were transferred from plastic pots to the respective Petri dishes (one insect per Petri dish) and placed randomly in an acclimatized room (24 ± 1 °C; 60 ± 10% RH, and 12:12 L:D). Observations of each insect's behaviour during ecdysis were made. As this usually occurs in the early morning, each insect was observed in its respective Petri dish every morning for a maximum of 40 minutes or until underwent and descriptions of behaviour during moulting were taken (control: N=15; infected: N=15). Some sequences of behaviours were recorded by a cellphone camera and representative videos from each treatment are supplied (Videos S1 and S2).

To compare the times spent to complete ecdysis in the two treatments, we built a GLM with a Gaussian distribution using *time to complete ecdysis* as the response variable, and *treatment* as the explanatory variable. We analyzed the residuals to check for distribution suitability and fit in the model. Four ecdysis-related behaviours were observed and are described fully in the Results: splitting the integument from within along the ecdysial line, performing soft body contractions, pulling and pushing its legs until they are released from the old cuticle, and releasing itself from the old cuticle. To compare the time that the insects spent in each behaviour, we built GLMs with Gaussian distributions using each of the cited variable as response variable (one at a time), and *treatment* as explanatory variable. We analyzed the residuals to check for distribution suitability and fit in the model. Statistical analysis was conducted using the software R (R Core Team 2019).

2.4. Ethics statement

All experiments using mice were approved by the Ethics Committee in Animal Experimentation (CEUA/FIOCRUZ) under the protocol number LW-8/17 and were performed in accordance with FIOCRUZ guidelines on animal experimentation.

3. Results

3.1. Experiment I – Infection with *Trypanosoma rangeli* caused mortality, and defective and delayed moulting

The cumulative mortality of infected insects was 6% higher than that of uninfected insects (infected: 30 ± 5.7%; control: 5 ± 5%; mean ± se; $\chi^2 = 12.73$; d.f. = 3, N = 32; $P < 0.01$; Fig. 1a). There were considerably more defective moults in infected than uninfected insects (infected: 50 ± 4.1% *vs.* control: 5 ± 5%; mean ± se; $\chi^2 = 5.88$; d.f. = 3; N = 32; $P < 0.01$; Fig. 1a). There was no effect of infection on the proportion of insects that did not initiate moult (infected: 5 ± 5% *vs.* control: 0 ± 0%; mean ± se; $\chi^2 = 2.68$; d.f. = 3; N = 32; $P < 0.01$; Fig. 1a). Infection reduced successful moulting to 5th instar ten-fold (infected: 10 ± 5%; control: 90 ± 10%; mean ± se; $\chi^2 = 32.69$; d.f. = 3; N = 32 $P < 0.01$; Fig. 1a). Survival over time was also higher for infected insects compared to controls: the median survival times after infected insects fed until repletion were 50 days (log-rank test: $\chi^2 = 8.6$; d.f. = 1, N = 40 $P < 0.01$; Fig 1b). The median lethal time for uninfected insects could not be estimated because the mortality after 60 days was less than 50%. Additionally, moulting took 2.5 times as long for infected *versus* uninfected insects, since infected insects median moulting time is on day 43 and control median moulting time is on day 18 (log-rank test: $\chi^2 = 627$; d.f. = 1, N=40; $P<0.001$; Fig. 1c).

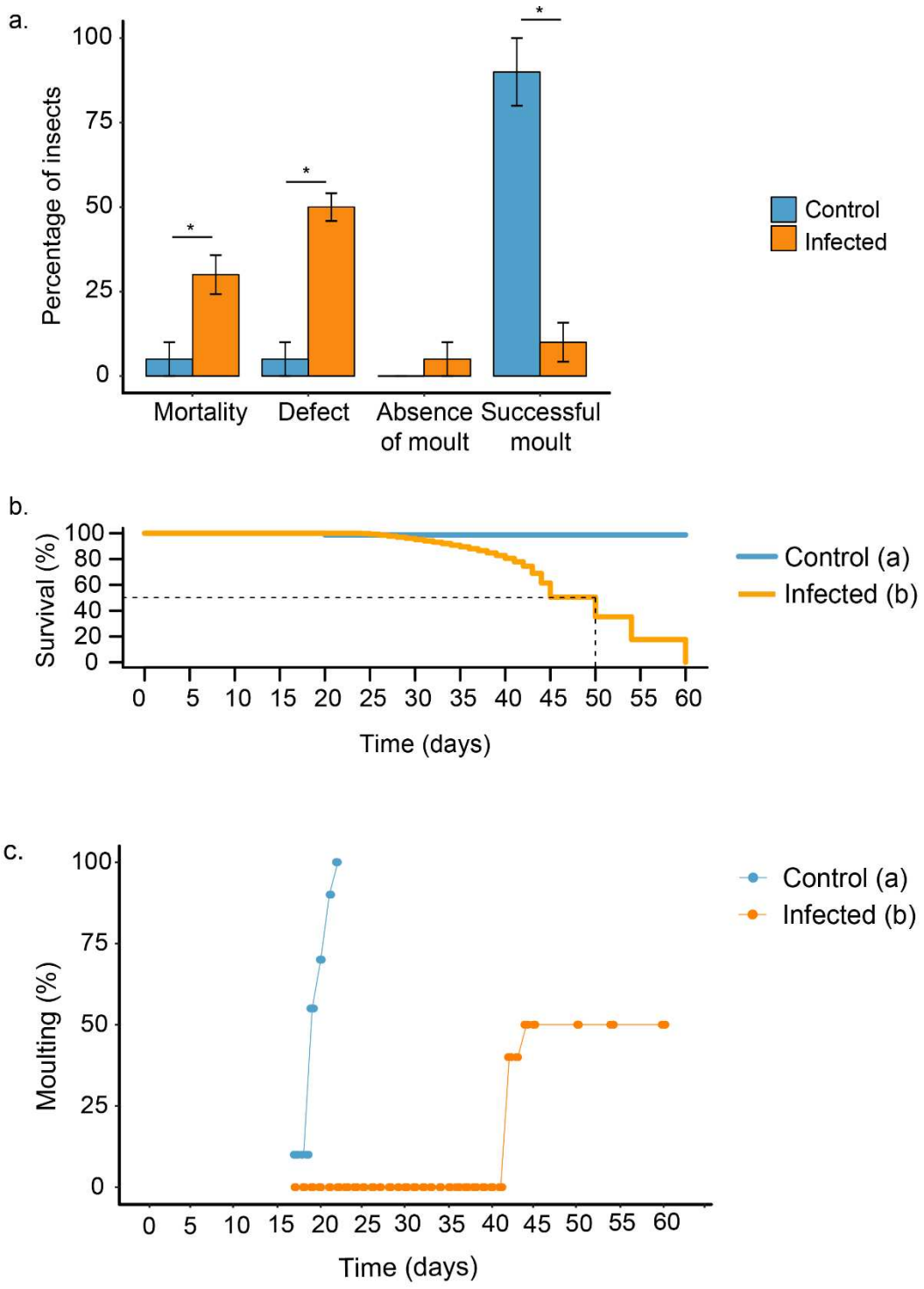

Fig. 1. Effect of infection by *Trypanosoma rangeli* on moult and survival from 4th to 5th instar of *Rhodnius prolixus* nymphs**.** a**.** Cumulative percentages of insects that moulted with visible morphological defects, died during moult, did not moult or successfully moulted; data are means ± SE; $P<0.01$). b**. S**urvival (%) of *Rhodnius prolixus* from the day ecdysis started to 60 days; dashed line represents the median lethal time of infected insects; different letters represent significant differences at α = 5%. c. Time required by 4th instar nymphs of *Rhodnius prolixus* to reach 5th instar; different letters represent significant differences at alfa = 5%.

3.2. Experiment II – Insects infected with *Trypanosoma rangeli* spent more time in ecdysis-related behaviours than uninfected insects

We observed the behaviours of thirty 4th instar nymphs (fifteen from each treatment) during ecdysis. Representative videos of the moment of ecdysis from each treatment are supplied (Videos S1 and S2). An overall description of the process of ecdysis follows:

For both infected and uninfected insects we observed the same ecdysis-related behaviours, when an individual is ready to escape from the old cuticle, it stays motionless for a while. Then, gentle muscular contractions begin to take place within the body, forcing it upward so that pressure is brought to bear on the dorsal surface of the ecdysial line (or dorsum-median line). Soon the integument begins to split along the ecdysial line, commencing above the prothorax and extending back to the abdomen. The body is then slowly withdrawn from the old cuticle by a series of soft body contractions. The legs are then withdrawn, by pulling and pushing and with further gentle body contractions and finally the tip of the abdomen emerges. On freeing itself from the old cuticle, insect locomotion is reduced until the new cuticle is melanized. Some insects present hind leg defects (such as curved tibias), but infected insects in particular have more defective moults (supplementary material - video S1 and video S2). Insects that spent more time to finish ecdysis normally died imprisoned within the old cuticle, which happened more often in infected insects.

To allow us to quantify differences between the treatment groups, we divided the process of ecdysis (above) into four main sequential phases (i to iv), according to key distinguishable behavioural features: (i) *ecdysial line disruption* (which we considered as the time that the insect spent splitting the integument from within along the ecdysial line), (ii) *motionless* (which we considered as the time that the insect stays performing a series of soft, almost imperceptible, body contractions), (iii) *leg liberation from the old cuticle* (which we considered as the time that the insect spends pulling and pushing its legs, one by one until they are released from the old cuticle) and (iv) *exuvium release* (which we considered as the time that the insect spends releasing itself completely from the old cuticle, now an exuvium) (Table 2).

Ecdysis of infected nymphs took 22.71 minutes *versus* 14.82 minutes for uninfected insects (infected: 22.71 ± 1.26min; control: 14.82 ± 0.75min; mean ± se; $F_{(1,28)} = 32.21$, $P < 0.001$; Fig. 5a, Table S1). Infected insects spent twice as long as controls did on *ecdysial line disruption* (infected: 6.76 ± 0.32min; control: 3.34 ± 1.01min; $F_{(1,28)} = 10.34$, $P < 0.001$; Fig.

5b) and half again as long on *exuvium release* (infected: 11.72 ± 0.21min; control: 7.05 ± 0.62min; mean ± se; $F_{(1,25)} = 48.40$, $P < 0.001$; Fig. 5e). However, there were no differences in time that insects spent *motionless* (infected: 1.52 ± 0.15min; control: 1.52± 0.16min; mean ± se; $F_{(1,27)} = 0$, $P > 0.05$; Fig. 5c) or *when releasing the legs* (infected: 4.57 ± 1.41min; control: 3.73 ± 0.26min; mean ± se; $F_{(1,26)} = 0.34$, $P > 0.05$; Fig. 5d.

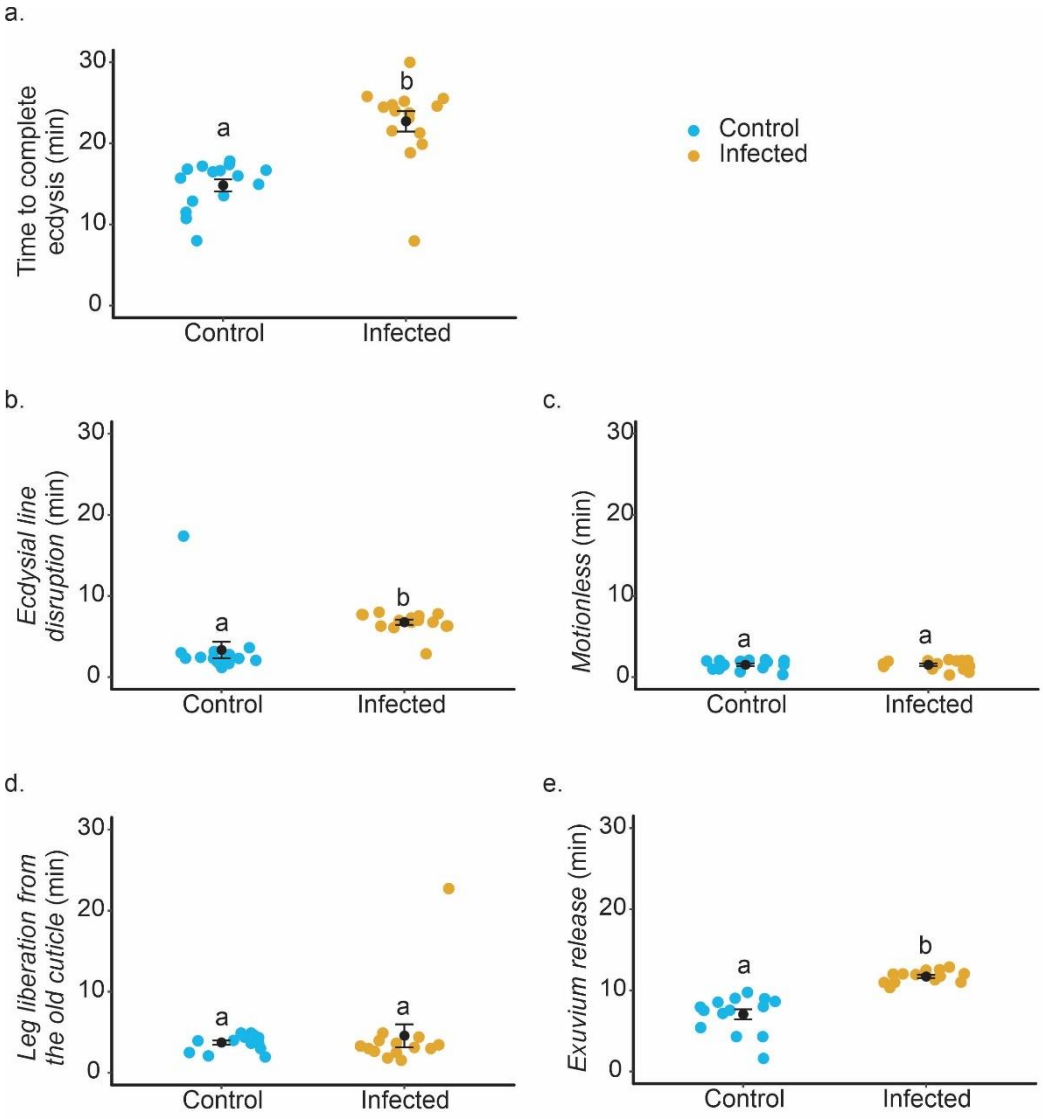

Fig. 2. Ecdysis-related behaviours of 4th instar *Rhodnius prolixus* nymphs that were uninfected (control) or infected with *Trypanosoma rangeli*. N=15 (a) Time spent to complete ecdysis ($F_{(1,28)} = 32.21$; $P < 0.001$). We represent time (min) spent on the following four main sequential behaviours during ecdysis: (b) *ecdysial line disruption* (which we considered as the time that the insect spent spliting the integument from within along the ecdysial line) ($F_{(1,28)} = 10.34$; $P < 0.001$), (c) *motionless* (which we considered as the time that the insect stays performing series of soft body contractions that are almost imperceptible) ($F_{(1,27)} = 0.00$; $P > 0.05$), (d) *leg*

liberation from the old cuticle (which we considered as the time that the insect spent pulling and pushing its legs, one by one until they are released from the old cuticle) ($F_{(1,26)} = 0.34$; $P > 0.05$) and (e) *exuvium release* (which we considered as the time that the insect spent releasing itself completely from the old cuticle, now an exuvium) ($F_{(1,25)} = 48.40$; $P < 0.001$). Data are means ± SE. Different letters represent significant differences at α = 5%.

4. Discussion

Insects infected by pathogenic microorganisms can show high mortality during moulting, but this phenomenon has not yet been fully documented. Here we documented this in such a way as to allow us to raise hypotheses about the mechanisms, focusing especially on behaviour. Using the triatomine *Rhodnius prolixus* and the protozoan parasite *Trypanosoma rangeli* as a model system, we quantified mortality during moulting and investigated alterations on behaviour during ecdysis.

Overall, we demonstrate that infected insects presented 6-fold higher mortality during their moulting process than uninfected insects and spent 2.5 times longer to reach the next instar. These results corroborate studies that also show high mortality of infected insects during moulting (Grewal, 1957; Watkins, 1971; Elliot et al 2002, 2015; Rodrigues et al., 2016). It is salient to highlight that we used the same strain of insect and parasite isolate used in the study of Rodrigues et al. (2016). Furthermore, once moulting had begun, there was an increase in time to complete ecdysis for infected insects.

Besides *T. rangeli* increases insect mortality, infection with this parasite delays moult in *R. prolixus*, and this result corroborates other studies that also reported a delayed moult in infected organisms. For instance, *T. cruzi* infection delayed moulting of of *R. prolixus* 2nd instar nymphs, by more than 10 days (Elliot et al., 2015). Similarly, *Metarhizium* infection delayed moulting of *Schistocerca gregaria* a few days compared with controls, when the locusts were allowed to thermoregulate away from the pathogen's ideal temperatures (Elliot et al., 2002). Thus, as quite disparate pathogens hinder insect development in at least two systems. There may be some common effect of infection leading to this delay, as quite disparate pathogens decrease insect development in at least two systems. We suggest cuticular thickness (which is directly related to cuticle development) and nutrient availability may explain this lengthened moult. We propose that the lack of fundamental macronutrients, such as carbohydrates or proteins can limit cuticle deposition and so impact insect development (Lake and Friend, 1968).

Infected insects showed more defective moults, and absence of moulting. Defective moults were characterized by tibial deformation, mainly (e.g., Watkins, 1971). For infected locusts, those that died during moulting ranged from individual that had only begun to shed the cuticle to individuals that remained with the cuticle attached (Elliot et al., 2002). In that case, death occurred due to starvation resulting from impaired mobility (SLE pers. obs.), something that would take a considerable length of time in triatomines. Also, it has been shown that some triatomines (*R. prolixus)* infected by *T. rangeli* were unable to moult while others died (Grewal, 1957). Possible mechanisms explaining deformation and absence of moult in infected insects can be related to vitamin deficiencies (Watkins 1971; Grewal 1957; Tobie 1970). As we know, under infection, pathogens may compete with their hosts for nutrients in the host heamolymph, impacting insect growth (Xia, Clarkson and Charnley, 2002). Such mechanism needs to be study in more detail.

Once moult had begun, infected insects spent longer in the first and last behavioural components (*ecdysial line disruption* and *exuvium release*, respectively) of the ecdysis process than did control insects. For the two middle sequential behaviours (*leg liberation from the old cuticle* and *motionless*) there were no differences between treatments. The long time to finish ecdysis possibly impacts insect survival since the new cuticle could start sclerotizing before finishing ecdysis, leading to insect death. Basically, the processes occur in a sequential manner, each step occurs at its own specific sequence. The fact of the insect is unable to complete ecdysis can be related to the swallowed air, as infected insects, unlike uninfected, cannot get the essential air to exert pressure on and burst the old cuticle (Watkins, 1971; Reynolds, 1980). The reason why insects cannot capture oxygen adequately is unknown, so it should be interesting to verify thracheal morphology to look for possible effects of parasite infection.

Another explanation for the long time taken to finish ecdysis is the hormonal alterations that can be caused by the infection. It has been shown that deletion or silencing the ecdysis-triggering hormone (ETH) in *Drosophila* causes deformities after ecdysis and lethality (Park et al., 2002; Lenaerts et al., 2017) – insect deformities include more vulnerability to predation and abiotic factors (Gullan and Cranston, 2014). For instance, silencing of ETH (which is a hormone that triggers a behavioral sequence critical for shedding of the old cuticle in arthropods) and its receptor resulted in lethality during ecdysis for *Schistocerca gregaria* (Lenaerts et al., 2017). This highlights the hormone's crucial role in moulting success. It is possible that *T. rangeli* causes silencing of ETH (Roller et al, 2010), which may ultimately cause an increase in time to escape the old cuticle.

Moulting is a complex process that involves hormonal, epidermal and cuticular changes, which can be impacted under pathogenic infection circumstances, causing insect death. This death can be related to failure to successfully complete ecdysis, and moulting defects. We have quantified the moulting-related mortality in diseased insects, and we suggest insect mortality is influenced by the time taken to complete ecdysis. Our results show adaptive relationship between insect and pathogen, such as triatomines and *Trypanosoma*. The delay in moulting may be adaptive because parasite infection delays the insect's next blood meal, as this only occurs after the insect has moulted as suggested by Rodrigues et al. (2016). Such a delay allows the parasite to complete its development cycle, favoring parasite transmission. It may even be the case that debilitating the insect during or after moulting could promote subsequent transmission of the pathogen – if cannibalization is a possible route or if the inability to walk or fly means that an insect falls to the ground, where a fungus can be found in the soil and be better able to sporulate and infect new hosts. Moreover, as we show here, focusing on ecdysis in studies of infection must be important, but it may be an undereported phenomenon.

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Supplementary material

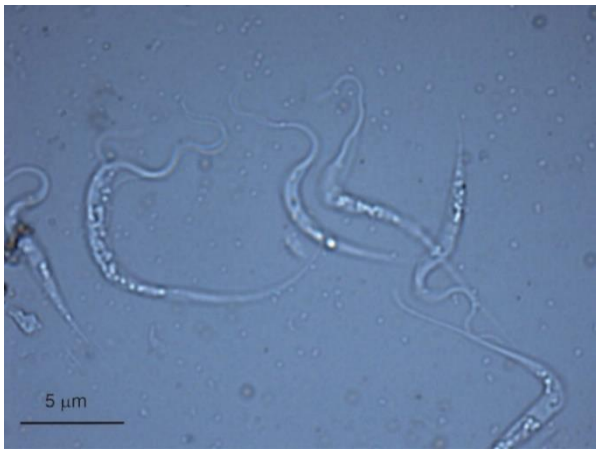

Fig. S1. Confirmation of haemolymph infection of 4th instar *Rhodnius prolixus* nymphs that had been infected with *Trypanosoma rangeli*. The samples were examined under a light microscope (100×). Shown are X stages of the parasite *T. rangeli*.

Table S1. Ecdysis-related behaviours of 4th instar *Rhodnius prolixus* nymphs that were infected or uninfected (control) with *Trypanosoma rangeli*. The time calculated in this table was based on the mean time of each behaviour type, respectively. We classified four main sequential behaviours during ecdysis: (i) *ecdysial line disruption* - which we considered as the time that the insect spent spliting the integument from within along the ecdysial line; (ii) *motionless* - which we considered as the time that the insect stays performing series of soft body contractions that are almost imperceptible; (iii) *leg liberation from the old cuticle* - which we considered as the time that the insect spent pulling and pushing its legs, one by one until they are released from the old cuticle; and (iv) *exuvium release* - which we considered as the time that the insect spent releasing itself completely from the old cuticle, now an exuvium. Representative videos of the moment of ecdysis from each treatment are supplied (Videos S1 and S2).

Behaviour type	**Treatment**	**Time (initial time-final time) (minutes)**
Ecdysial line disruption	Control	0 - 3.34
	Infected	0 - 6.76
Motionless	Control	3.35 - 4.87
	Infected	6.77 - 8.29
Leg liberation from the old cuticle	Control	4.88 - 8.61
	Infected	8.30 - 12.87
Exuvium release	Control	8.62 - 15.67
	Infected	12.88 - 24.60

Video recordings:

Video S1 – control insect:

https://drive.google.com/file/d/103PNoJ3qqqUnVVZOprUxtGr06bU_GRHt/view?usp=sharing

Video S2 – infected insect:

https://drive.google.com/file/d/1rr6S06m22tEdglnJfpn_4EddgzBDaVRY/view?usp=sharing

CHAPTER III

MOULTING-RELATED MORTALITY IN DISEASED INSECTS: POSSIBLE ROLES OF CUTICLE DEVELOPMENT AND ECDYSONE LEVELS

Article 3: Moulting-related mortality in diseased insects: possible roles of cuticle development and ecdysone levels

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Abstract

Insect development occurs through moulting, where the individual produces a new cuticle and sheds the old one, allowing the insect to grow and develop. Insects infected by pathogenic microorganisms can show increased mortality during moulting, but underlying mechanisms for this are as yet unknown. Using the model system of the triatomine bug *Rhodnius prolixus* (Hemiptera: Reduviidae) and the protozoan parasite *Trypanosoma rangeli*, here we investigate cuticular thickness and ecdysone levels as potential factors. We also investigated glycogen content in fat body, and midgut and hindgut morphology as possible factors increasing the development time of moulting as results of the microorganism infection. In line with previous reports, mortality of 4th instar *T. rangeli*-infected nymphs was 6% higher than that of uninfected insects. Histological analyses of cuticular show that sternite development over time was thinner in infected than uninfected insects. Levels of ecdysone were lower in infected insects compared to control. We suggest that cuticle thickness is related to the lengthened time to moult of infected insects and the low levels of ecdysone influences insect survival. The low glycogen content in fat body of infected insects can explain the delay in moulting found in Chapter 2. Infected insects seem not to have damage in the midgut epithelial cells, but the epithelium is apparently thicker than the control. Our results are a starting point for further studies on the moulting physiology considering an insect-pathogen interaction.

Keywords: cuticle, ecdysone, glycogen, infection, moulting, parasite, *Rhodnius prolixus*, triatomine, *Trypanosoma.*

1. Introduction

Insect growth is based on periodic moults, in which process a new cuticle is produced to replace the old (Wigglesworth, 1934; Chapman, 2013). The process of moulting is complex, involving division of epidermal cells, synthesis of a new cuticle, partial digestion and resorption of the old cuticle, neural stimulus and endocrinal regulation (Wigglesworth, 1934; Chapman, 2013). However, when infected with pathogenic microorganisms, insects such as locusts (Orthoptera: Acrididae) and triatomines (Hemiptera: Reduviidae) show elevated mortality during moulting (Grewal, 1957; Watkins, 1971; Elliot et al. 2002, 2015; Rodrigues et al., 2016). For instance, *Schistocerca gregaria* (Orthoptera: Acrididae) infected by the fungal pathogen *Metarhizium acridídum* (Ascomycetes: Hypocreales) died during moulting even when able to use a behavioural fever response (Elliot et al., 2002). Similarly, more than 50% of the mortality found in *Rhodnius prolixus* (Hemiptera: Reduviidae) infected by the protozoan *Trypanosoma rangeli* (Kinetoplastida, Trypanosomatidae) occurred during ecdysis (Rodrigues et al., 2016). In both cases, diverse morphological defects were visible, and insects were often unable to escape from their old cuticles (exuvia), although this was only recorded as an observation in each case. Thus, there are at least two host-parasite systems–and disparate ones–where we can find high mortality during insect moulting. Despite the limited data we have to date on this phenomenon, it indicates that there is something of potential interest happening across systems. To understand it properly, it is necessary to examine the biological mechanisms underlying the high mortality during moulting of diseased insects.

For clues as to the mechanisms involved in this phenomenon, we can examine previous studies that deal with moulting. Ecdysteroids are hormones that are responsible for triggering cuticle formation (Chapman, 2013). It is known that pathogens may be able to affect the developmental process of their insect hosts negatively by inactivating these hormones (e.g., in lepidopteran larvae infected with a fungal pathogen; Zhu et al., 2021). Meanwhile, defects in cuticle formation may be related to limited availability of nutrients such as amino acids and vitamins, and this can occur due to infection with parasites. For example, concentration of some amino acids in the haemolymph of *R. prolixus* is decreased in the presence of *Trypanosoma rangeli* and this interferes with insect moulting (Ormerod, 1967). Also, macronutrients such as carbohydrates are used by insects as energy sources for fundamental biological processes. For instance, locusts that fed on a diet rich in carbohydrates have lower mortality from a fungal infection than the insects that fed on a diet rich in protein; in relation to time, infected locusts

that fed on a diet with low carbohydrate and high protein died faster than the insects that fed on a balanced diet (Graham, et al. 2014). These results can indicate the cost of such macronutrients for surviving an infection as well as for continuing the developmental process. Moreover, trehalose is another nutrient that, besides being used by the insect, can potentially be used by entomopathogenic fungi in the haemolymph by direct uptake (Lepidoptera – Xia et al, 2002). Meanwhile, in dipteran larvae infected with a parasitoid, glycogen is broken down into glucose, which helps the insect to fight the parasite (Ponton, et al. 2019). Based on these examples, it could be that nutrient availability influence insect normal cuticle formation, as this process needs resources (e,g, amino acids; Ormerold, 1967) for which the microorganism may be competing.

To address the question of why infected insects suffer higher mortality during moulting than uninfected insects (e.g. Watkins, 1971; Elliot et al. 2002, 2015; Rodrigues et al., 2016) we used *R. prolixus* infected by *T. rangeli* as a model system. *Rhodnius prolixus* has been used as a model because of the detailed knowledge available on its physiology (Wigglesworth, 1934; Guarneri and Lorenzo, 2021). This insect is also easy to maintain in the laboratory and can survive for months without food. However, when fed to repletion, the insect moults to the next instar (Wigglesworth, 1934), meaning that it is possible to control insect moulting experimentally. Moreover, this insect is one of the most important vectors of Chagas disease (Garcia and Azambuja 1991). Meanwhile, *Trypanosoma rangeli* is also a model to study infection in triatomines. The way these parasites infect the insect is similar to other entomopathogenic microorganisms that cross a barrier and infect the hemolymph.

We hypothesize that the negative effects of pathogenic microorganisms on insect moulting occur through impacts on development of the cuticle and alterations in levels of ecdysone. We also hypothesize that the delay in insect moulting reported previously from this system (Rodrigues et al., 2016) is due to delayed development of the cuticle. We suggest that such a delay is related to availability of glycogen for biological processes, and/or to nutrient absorption by the gut epithelium. To investigate these hypotheses, we examined (i) cuticle thickness and development, (ii) levels of ecdysone, (iii) storage of glycogen in fat body, and (iv) morphology of midgut and hindgut epithelium in uninfected and infected insects. With this, we hope to elucidate fundamental physiological processes that may explain mortality during moulting of insects infected by pathogenic microorganisms in general.

2. Material and Methods

2.1. Triatomines, parasites and life stages

We obtained *Rhodnius prolixus* from the laboratory at the René Rachou Institute (FIOCRUZ, Minas Gerais, Brazil). The colony is maintained under laboratory conditions (26 ± 1°C; 65 ± 10% RH; and natural illumination cycle). For all of our experiments, 4th instar nymphs were fed on mice that we anesthetized with an intraperitoneal injection that contains ketamine (150 mg/kg; Cristália, Brazil) and xylazine (10 mg/kg; Bayer, Brazil).

We used the 'Choachi' strain of the *T. rangeli*. It was isolated from naturally infected *R. prolixus* (Schottelius, 1987). For infection of the triatomines, we used epimastigote forms of the parasite that were cultured by weekly passages at 27°C in liver-infusion tryptose (LIT) medium, and supplemented with 100 units/ml of penicillin, and 100 mg/ml of streptomycin.

The development of triatomines includes eggs, five nymphal instars and adults. These insects are obligatory hematophages and need to feed on blood during all phases of their life cycle. After *R. prolixus* feeds on *T. rangeli*-infected mammals, the trypomastigote forms that were ingested with the blood meal reach the midgut and differentiate into epimastigote forms – now it can colonize the insect intestine and cross its epithelium; now, in the haemolymph, the parasites multiply and reach the salivary glands - here epimastigotes forms differentiate into metacyclic trypomastigote forms; these are the forms that are transmitted to a vertebrate host in the next blood meal of the insect (Tobie, 1970; D'Alessandro, 1976; Hecker et al., 1990).

2.4. Infections of mice and triatomines

For oral infection, female Swiss Webster mice were used. They were infected with *T. rangeli* through the bite of 5th instar nymphs infected by epimastigote forms in their salivary glands. For the following experiments, mice were used at 14 days post-infection.

Fourteen-day old 4th instar nymphs were infected according to Guarneri et al. (2020). For intestinal infection, the nymphs were separated and fed on healthy or *T. rangeli*-infected mice. Through this feeding protocol, each insect was allowed to ingest approximately 50–150 trypomastigotes (Rolandelli et al 2021). Rates of *T. rangeli* migration across the midgut are variable in *R. prolixus* (2–50%; Guarneri et al. 2020), and the parasite does not always invade the hemocoel in the natural course of the insect infection (Ferreira et al., 2010). So, after

infection of the midgut, we also inoculated parasites into the insect hemocoel, guaranteeing the production of metacyclic forms and standardizing parasite densities in the haemolymph (Guarneri, 2020). For hemocoel inoculation, *T. rangeli* epimastigotes were obtained from cultures, and resuspended in sterile phosphate buffered saline (PBS; 0.15 M NaCl in 0.01 M sodium phosphate, pH 7.4) at a concentration of 5×10^4 epimastigotes/ml of PBS. After a week of intestinal infection by *T. rangeli*, the nymphs were inoculated through the thoracic pleura with 1 µl of PBS (Control) or PBS + the parasite suspension, using a microliter syringe (50 µl). We used a dispenser (model 705, Hamilton Company, NV, USA) coupled to the syringe (Hamilton Company, NV, USA, needle 13mm × 3.3mm; ½") to increase the precision of the inoculum. Twenty-four hours after inoculation, both groups of insects were blood-fed to repletion on healthy mice, in order to trigger the moulting process: this procedure leads to ecdysis occurring after *ca*. 15 days in 4th instar nymphs. Haemolymph infection (parasitemia) was confirmed by cutting the terminal segment of a hind leg and collecting a drop of haemolymph on a microscope slide. The samples were examined under a light microscope (100×) (Fig. S1). Parasitemia was thus confirmed immediately after each experiment.

2.5. Experiment I – Cuticular thickness and development

To analyze insect cuticular thickness and development, control and infected 4th instar nymphs of *R. prolixus* were transferred to Petri dishes (60mm × 15mm). We transferred six insects to each Petri dish and there were five Petri dishes for each treatment. The Petri dishes were randomly placed and kept in a room under controlled conditions (24 ± 1 °C; 60 ± 10% RH, and 12:12 L). For cuticular histological analyses, three insects from three different Petri dishes were collected for each treatment on days 9, 11, 13 and 17 and for infected insects only on days 19 and 42 – note that ecdysis normally occurs at ca. 15 days after feeding for uninfected insects but is delayed in infected insects (e.g., Rodrigues et al. 2016; Chapter 2). For each time point, the three insects collected from each treatment were cryo-anesthetized at -4°C, dissected in saline solution (0.1 M NaCl, 0.1 M KH2PO4 and 0.1 M Na2HPO4), and separated into different fragments from the thorax (where we find the ecdysial line), abdominal sternite and tergite, under a stereomicroscope. Then, insect fragments were transferred to Zamboni's fixative solution for at least 24h. We dehydrated the samples in a graded ethanol series, and gradually embedded them into resin (resin: ethanol = 1:2, for three days; 1:1, for three days; 2:1, for three days, and pure resin, for three days). We took sections with a microtome and slides

(3µm thick) were stained with hematoxylin and eosin. We photographed all samples under a light microscope (40×). All images were prepared using CorelDRAW, and cuticle measurements made using Image Pro Plus. We randomly measured three different parts of each cuticular image, and then calculated the mean for each image (N=6).

To compare cuticular thickness from each treatment according to time, we built a general linear model (GLM) with a Gaussian distribution using *cuticular thickness* as the response variable and *treatment* and *time* as explanatory variables. We analyzed the residuals to check for distribution suitability and fit in the model. Statistical analysis was conducted using the software R (R Core Team 2019). Additionally, to compare *cuticular thickness* from each treatment before and after ecdysis, we built a general linear model (GLM) with a Gaussian distribution using *cuticular thickness* as the response variable and *treatment* as explanatory variable. We analyzed the residuals to check for distribution suitability and fit in the model.

2.6. Experiment II – Levels of ecdysone

To analyze the levels of ecdysone (20E) in control and infected *R. prolixus* through time, we prepared a stock solution of commercial 20E in methanol to a final concentration of 1 mg/ml (Sigma-Aldrich for both chemicals.) Final concentrations of 0.1, 0.2, 0.3, 0.5, 0.8, 1.0, 1.5 and 2 ng/ml were prepared in methanol and stored at -80˚C. These concentrations were used to construct the calibration curve by plotting the area versus 20E concentrations. The equation of this curve was used to calculate the concentration of 20E in our samples, based on the area previous calculated in the DataAnalysis program (version 4.0 (Bruker Daltonics, Germany). After this, we calculated the weight in nanograms and subsequently converted this to picograms.

Control and infected 4th instar nymphs of *R. prolixus* were transferred to Petri dishes (60mm × 15mm). We transferred one insect to each Petri dish. There were six Petri dishes for each treatment and for the following days after insects had fed until repletion – 7, 9, 11, 14 & 18 days for both control and infected insects, plus 42 days for the infected treatment (controls had already moulted). The Petri dishes were randomly placed and kept at 24 ± 1 °C; 60 ± 10% RH and 12:12 L. We stored six insects per treatment, from 7, 9, 11, 14, 18 and 42 days (on day 42 - infected insects only) in an ultrafreezer (-80°C). To prepare the samples, each insect was weighed in 2ml microtubes. After this, 700 µl of methanol (at ambient temperature of 25˚C ± 2) was added to each microtube and the samples were homogenized 30 times using pestles to

extract the 20E. The sample was centrifuged at 10,000 g for 10 min. The supernatant (upper methanol layer) was then transferred by filtering this into glass vials, and then analyzed by liquid chromatography mass spectrometry.

Aliquots of 5µL were used for nano LC-MS analysis, using the nanoAcquity UPLC system (Waters, USA), containing a trap column and a capillary column (ProteCol C18 GHQ303 3.0µm - 300µm × 150mm), operating with a flow rate of 0.6uL/min. The samples were automatically injected into the mass spectrometer of the Ion Trap Amazon (Bruker Daltonics, Germany), operating in online mode, with the aid of a nanoESI ionization needle. In this step, the mobile phase solutions used for the gradient program were: (A) water and 0.1% formic acid (v/v) and (B) acetonitrile and 0.1% formic acid (v/v), with the gradient program consisting of a linear upward ramp from 2% to 50% of (B) over 20min, 50% to 98% of (B) over 10min, 90% of (B) over 20min, ramp down linear starting from 98% to 2% for 2 min., followed by 2% equilibration of (B) for 8 min, totaling 60 min of chromatographic run. The equipment was operated in positive mode and using the MRM (multiple reaction monitoring) scanning method to monitor the presence of ions with mass of 381.3 m/z, and fragments 371.1 m/z, 445,2 m/z, and 463.2 m/z, corresponding to the ecdysone molecule. Pure standards were used to construct a standard curve, and to determine the chromatographic retention time (RT=15min) and the masses and intensities of the fragments, as mentioned in the beginning of this section.

The data were acquired for 60 min in each LC-MS/MS analysis, with the acquisition of LC-MS data managed by the Hystar application, version 3.2 (Bruker Daltonics, Germany), the spectra being processed with the aid of the DataAnalysis application, version 4.0 (Bruker Daltonics, Germany), using default settings for metabolomics.

To compare the levels of 20E in each treatment, we built a GLM with a Gaussian distribution using *concentration of 20E* as the response variable, and *treatment* and *time* as explanatory variables. There was no interaction between *treatment* and *time*, so we removed the interaction from the model. We analyzed the residuals to check for distribution suitability and fit in the model. Additionally, to compare the levels of 20E before ecdysis, we built a GLM with a Gaussian distribution using *concentration of 20E* as the response variable, and *treatment* as explanatory variable. We analyzed the residuals to check for distribution suitability and fit in the model. Statistical analysis was conducted using the software R (R Core Team 2019).

2.7. Experiment III – Glycogen storage and midgut and hindgut epithelium morphologies

To verify if infection impacts glycogen storage or midgut and hindgut structures, both factors that we considered might affect insect cuticle formation, control and infected 4th instar nymphs of *R. prolixus* were transferred to Petri dishes – one insect in each Petri dish (60mm × 15mm). There were nine Petri dishes for each treatment. The Petri dishes were randomly placed and kept in a room with temperature and humidity controlled (24 ± 1 °C; 60 ± 10% RH, and 12:12 L). For fat body and midgut and hindgut histological analyses, three insects from different Petri dishes were collected for each treatment, before ecdysis (13 days after feeding until repletion, so ca. 2 days before ecdysis) and 12 hours after ecdysis (ecdysis occurred from 18-22 days for controls and 42-60 days for infected insects; for the analysis we used insects that had moulted on day 18 for control and on day 42 for infected insects) The insects collected from each treatment were cryo-anesthetized at -4°C, dissected in saline solution (0.1 M NaCl, 0.1 M KH2PO4, and 0.1 M Na2HPO4), and separated into fat body and midgut and hindgut, under a stereomicroscope. Each midgut and hindgut collected was separated in 4 parts, which we called as ventricles (V1 = ventricle 1, anterior midgut; V2 = ventricle 2, posterior midgut; V3 = ventricle 3, posterior midgut; V4 = ventricle 4, hindgut). Then, all insect fragments were transferred to Zamboni's fixative solution for at least 24h. We dehydrated the samples in a graded ethanol series, and embedded them in resin, then took sections with a microtome. Sections were placed on slides (3μm thick) and were stained with hematoxylin and eosin, while fat body sections were stained with periodic acid-Schiff: for this, coverslips were incubated in Periodic Acid Solution (PAS) and then Schiff's Reagent. We photographed all samples under a light microscope (40x).

For the glycogen analysis, we took three pictures for each sample of fat body and measured the pixel quantity (arbitrary units) (Viana, et al. 2018) using the software Gimp (Solomon, 2009). To compare glycogen quantity (arbitrary units) from each treatment, we built a GLM with a Gaussian distribution using *quantity of pixels* as the response variable, and *treatment* and *time* as explanatory variables. We analyzed the residuals to check for distribution suitability and fit in the model. Statistical analysis was conducted using the software R (R Core Team 2019).

2.8. Ethics statement

All experiments using mice were approved by the Ethics Committee in Animal Experimentation (CEUA/FIOCRUZ) under the protocol number LW-8/17 and performed in accordance with FIOCRUZ guidelines on animal experimentation.

3. Results

3.1. Experiment I – Infection with *Trypanosoma rangeli* prolonged time for insect cuticle formation

The cuticule of the sternite was thinner in infected than uninfected insects (Fig. 1a, Fig. S6a, Fig. S2, Table S1). However, for the tergite and thorax there were no differences in cuticular thickness (Fig. 1b,c, S6b,c, Fig. S3, Fig. S4, Table S1). Interestingly, cuticle from uninfected and infected insects has external protuberances that look like small stars (Fig. S5).

The sternite cuticle was thinner before and after ecdysis in the infected insects than in the controls (Before –infected: 12.22 ± 0.83 μm**;** mean ± se); control: 16.61 ± 0.62 μm; After – infected: 12.66 ± 0.32 μm**;** control: 17.40 ± 1.11 μm; mean ± se) ($F_{(1,26)} = 19.83$; $P < 0.01$; Fig. S6a). However, the cuticular thickness of the tergite, before ecdysis was lower in the infected insects than the control (infected: 15.25 ± 1.93 μm**;** control: 19.60 ± 1.23 μm; mean ± se); Fig. S6b, and after ecdysis the cuticular thickness was higher in the infected insects than control (infected: 22.46 ± 7.25 μm**;** control: 13.51 ± 0.22 μm; mean ± se) ($F_{(1,26)} = 4.49$; $P < 0.05$; Fig. S6b). There were no differences in the cuticular thickness of the thorax between the infected and control insects before ecdysis (infected: 13.85 ± 1.30 μm; control: 14.95 ± 1.25 μm; mean ± se); however, after ecdysis, the cuticular thickness of the thorax was higher in the infected insects than control (infected: 32.88 ± 3.41 (μm**);** control: 11.75 ± 2.42 μm; mean ± se) ($F_{(1,25)} = 27.78$; $P < 0.01$; Fig. S6c).

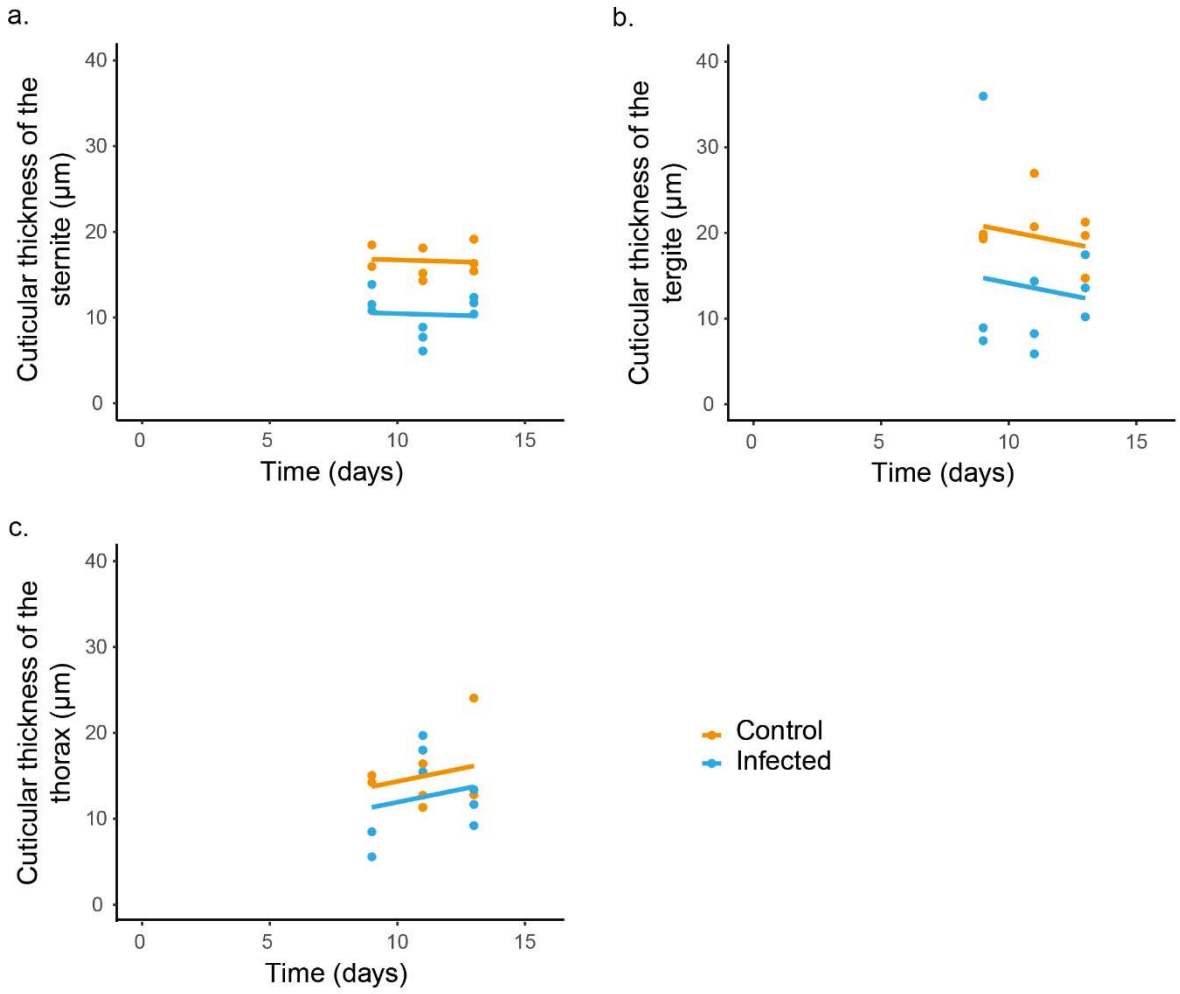

Fig.1. Cuticular thickness (µm) of sections of sternite (a), tergite (b) and thorax (c) of 4th instar *Rhodnius prolixus* nymphs that were uninfected (control) or infected with *Trypanosoma rangeli*, measured through time. We examined cuticles of control and infected insects from days 9, 11 and 13 (given feeding is usually followed by ecdysis ca. 15 days later). Note that lines are parallel as there was no interaction between time and infection status. Sternite: $F_{(1,18)}$=33.84, P< 0.001; tergite: $F_{(1,15)}$=3.21, P> 0.05; thorax: $F_{(1,14)}$=1.31, P>0.05. Additional statistical data are given in Table S1.

3.2. Experiment II – Infection with *Trypanosoma rangeli* reduced levels of ecdysone (20E)

Levels of ecdysone (20E) product ion 371 (pg/mL/mg) were higher along the days in infected insects compared to control ($F_{(1,18)} = 6.02$; $p < 0.05$) (Fig. 2a). Levels of ecdysone (20E) product ion 371 (pg/ml.mg) were lower in infected insects compared to controls before ecdysis (infected: 0.06 ± 0.03 (pg/ml.mg**);** control: 0.32 ± 0.07 (pg/ml.mg); mean ± se) ($F_{(1,18} = 5.96$; $p < 0.05$) (Fig. 2b). We detect ecdysone levels after ecdysis only in one sample, so we could not compare ecdysone levels after ecdysis.

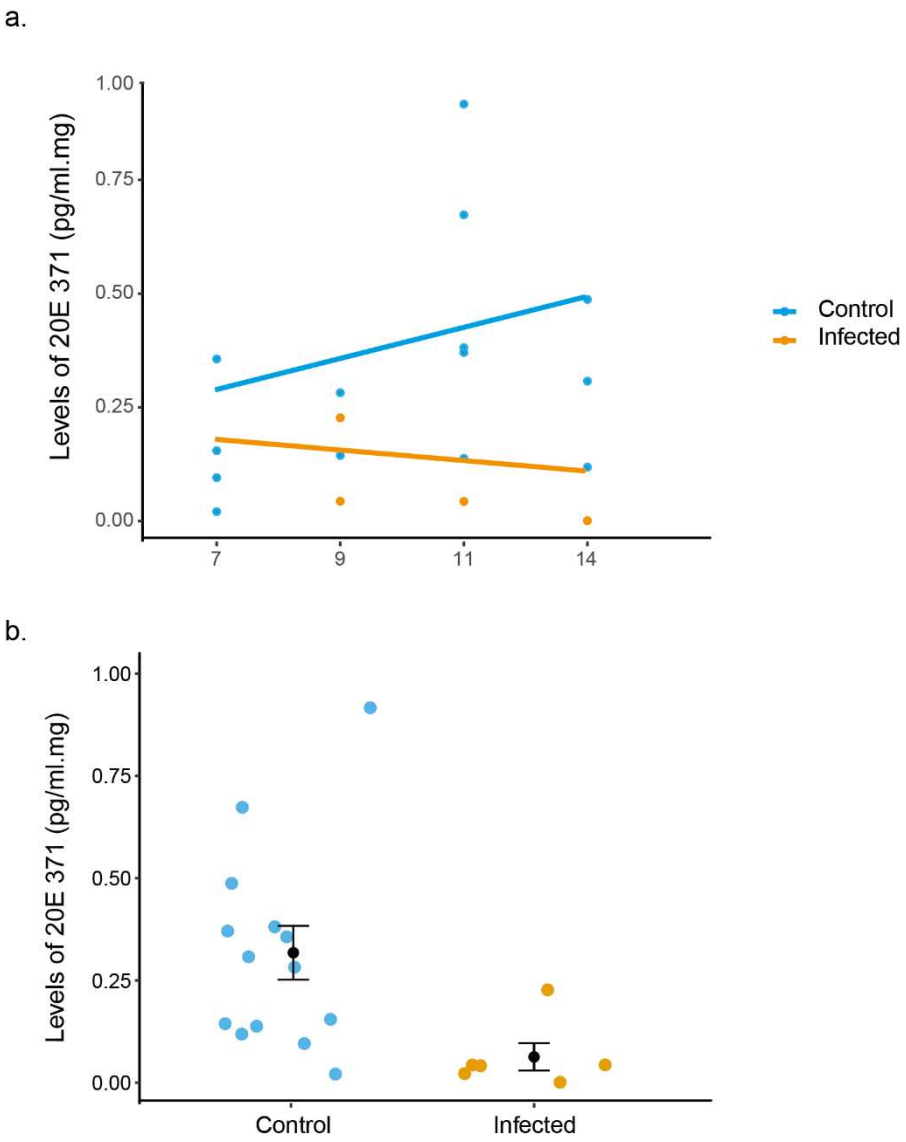

Fig. 2. Levels of the 20-hydroxyecdysone product ion 371 (pg/ml.mg) of 4th instar *Rhodnius prolixus* nymphs that were uninfected (control) or infected with *Trypanosoma rangeli*: (a) measured along the time - 7, 9, 11 & 14 days for both control and infected insects, and 42 days for the infected treatment (controls had already moulted) ($F_{(1,19)} = 7.86$; $p \leq 0.01$); and (b) measured before and after ecdysis ($F_{(1,20)} = 5.55$; $p < 0.05$).

3.3. Infection with *Trypanosoma rangeli* decreased glycogen storage in the fat body

Thirteen days before ecdysis, glycogen storage was 1.52-fold lower in infected insects than the controls (infected: 73,288.67 ± 24,063.12 arbitrary units; control: 111,324.22 ± 13,897.06 arbitrary units (pixels); mean ± se) ($F_{(1,4)} = 42.50$; $P < 0.01$; Fig. 3a, 3b (A, B)). However, twelve hours after ecdysis, there was no difference in glycogen storage between the control and infected insects 12 hours after ecdysis (infected: 35,449.00 ± 17,023.52 arbitrary units; control: 12,624.22 ± 6,005.91 arbitrary units; mean ± se) ($F_{(1,4)} = 1.98$; $P > 0.05$; Fig. 3a, 3b (C, D)).

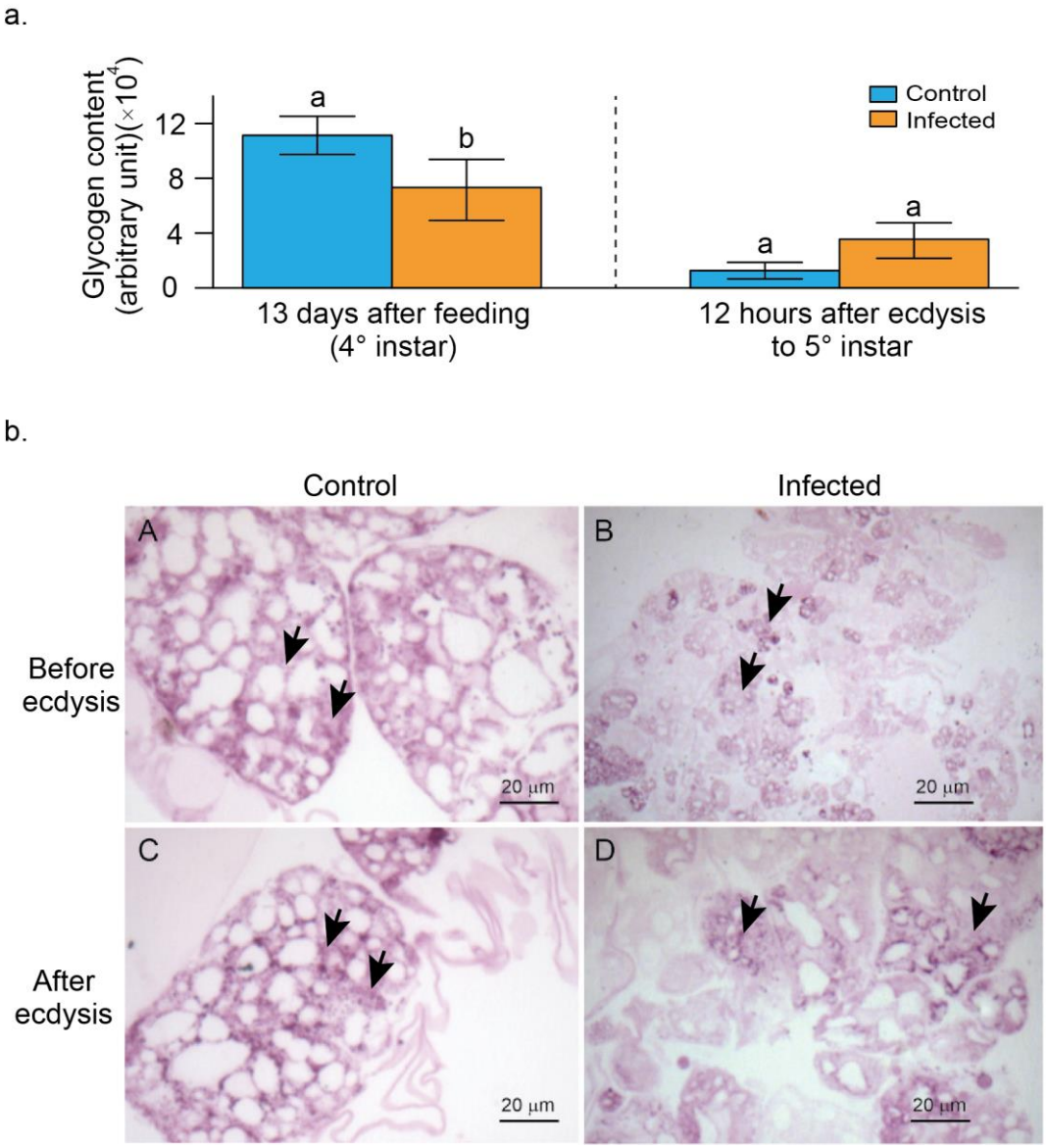

Fig. 3. Analysis of glycogen storage in the fat body of 4th instar *Rhodnius prolixus* nymphs that were uninfected (control) and infected with *Trypanosoma rangeli*, before and after ecdysis. (a). Quantification of glycogen (measured by density of pixels – arbitrary units ($\times 10^4$)): (i) in control and infected insects, 13 days after feeding to repletion (i.e. immediately prior to ecdysis on day 18 for uninfected insects) ($F_{(1,4)}$= 42.50; $p<0.01$), and (ii) 12 hours after ecdysis of 4th to 5th instar (which occurred on day 18 for control insects and day 42 for infected insects) ($F_{(1,4)}$= 1.98; $p>0.05$). Data are means ± SE. Different letters represent significant differences at α = 5%. (b) Histology of the fat body of control and infected insects, 13 days after feeding to repletion (i.e. immediately prior to ecdysis on day 18 for uninfected insects) (A, B) and 12 hours after ecdysis from 4th to 5th instar (which occurred on day 18 for control insects and day 42 for infected insects) (C,D). Gut sections (3 µm) were placed on slides and stained with periodic acid-Schiff (PAS). Glycogen is the purple granules in the images (some are indicated by black arrows). Dark and light granules indicate strong and weak positive reaction for glycogen presence, respectively. In A and C there are more purple granules than in B and D, meaning that

there is more glycogen available in control than infected insects. Scale= 20 μm. Images were taken under a light microscope (40×).

3.4. Infection with *Trypanosoma rangeli* did not impact midgut and hindgut morphologies, but may have increased epithelium thickness

The anterior and posterior midgut, and hindgut of *R. prolixus* were cut into different parts: ventricle 1, 2, 3 and 4 (V1 = ventricle 1, anterior midgut; V2 = ventricle 2, posterior midgut; V3 = ventricle 3, posterior midgut; V4 = ventricle 4, hindgut) (Fig. S7) at 13 days after feeding until repletion and 12 hours after ecdysis. For infected and control insects, samples from V1, V2, V3 and V4 present simple epithelium, containing a layer of cells with a single nucleus. Samples from V1 and V2 present columnar cells, while V3 and V4 present cubic cells. According to the morphology of V1, V2, V3 and V4, of infected and uninfected insects, 13 days after feeding until repletion and 12 hours after ecdysis, infected insects seem not to have damage in the midgut and hindgut epithelium (Fig. 4, 5). However, the epithelia of V1 and V2 of infected insects are apparently thicker than the control and also appear to have more basophilic granules in the cells than the control (for V1).

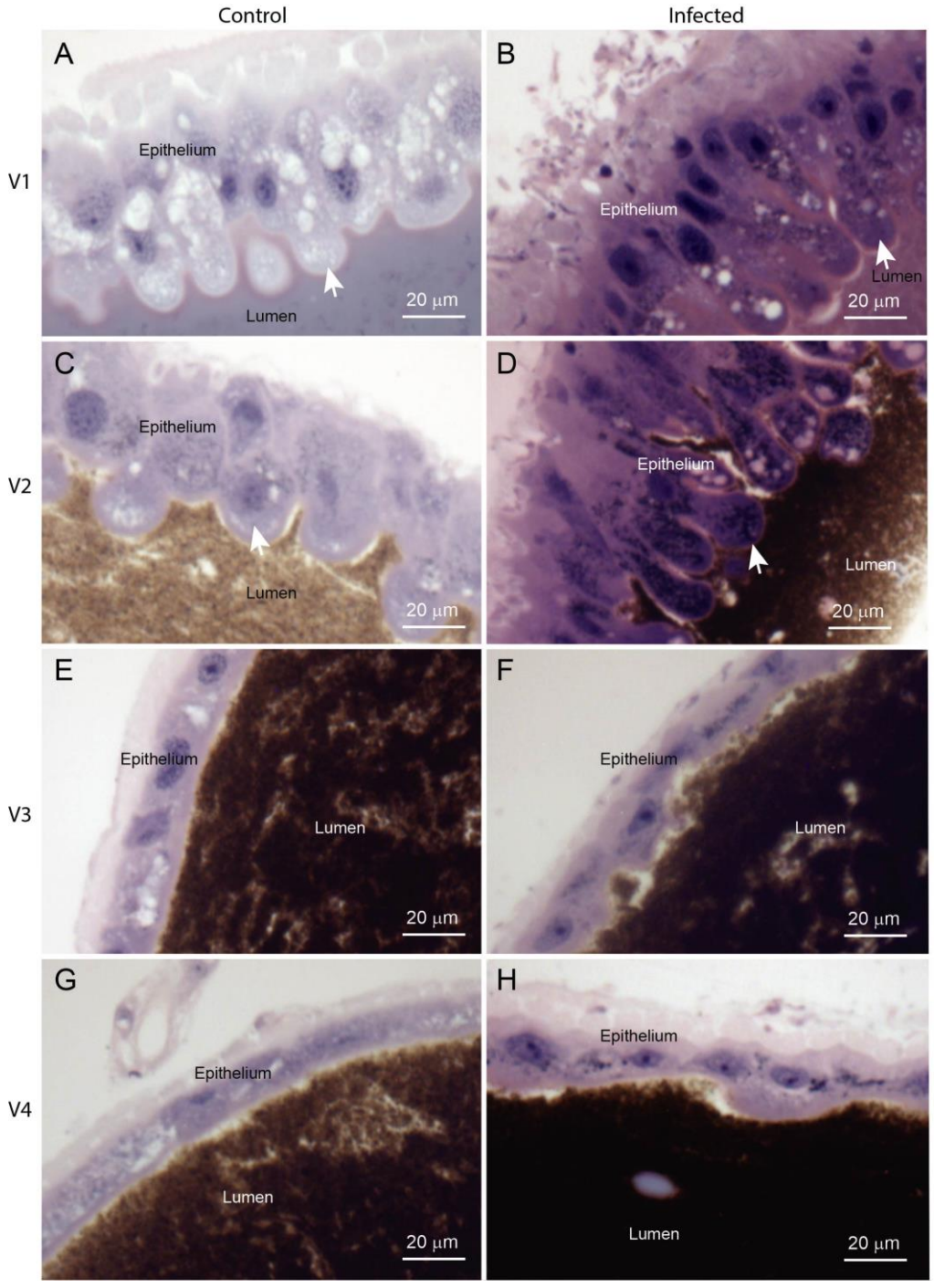

Fig. 4. Internal morphology of the midgut and hindgut of 4th instar *Rhodnius prolixus* nymphs that were uninfected (controls) or infected with *Trypanosoma rangeli*, 13 days after feeding to repletion. Shown are control (A, C, E, G) and infected (B, D, F, H) internal morphology from V1 (A, B), V2 (C, D), V3 (E, F), V4 (G, H). Note that V1 and V2 of the infected insects are apparently thicker than the control, and, in V1, infected insets appear to have more basophilic granules than the control. V1 = ventricle 1, anterior midgut; V2 = ventricle 2, posterior midgut; V3 = ventricle 3, posterior midgut; V4 = ventricle 4, hindgut. Scale= 20 μm. Slides were photographed under a light microscope (40×).

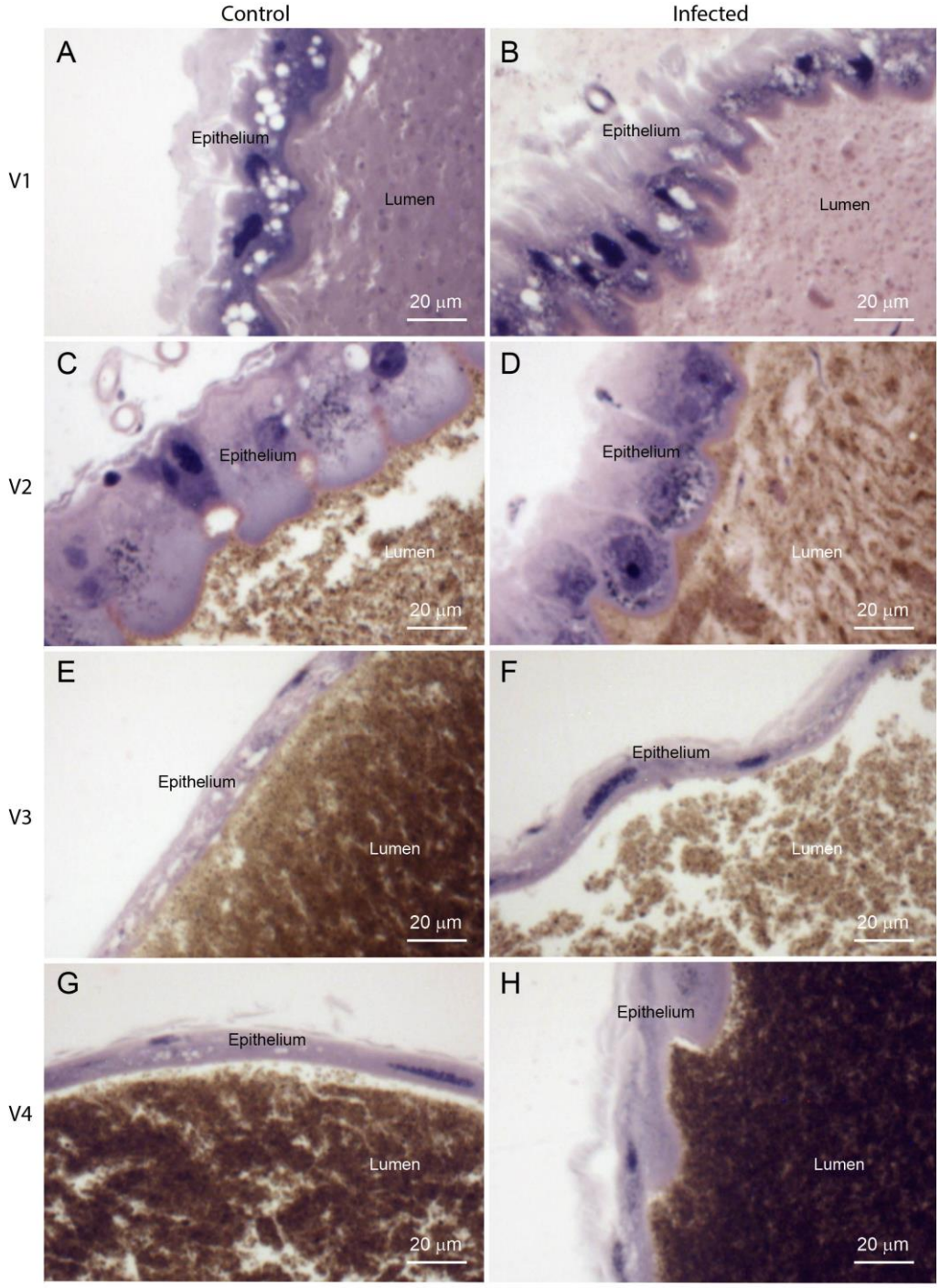

Fig. 5. Internal morphology of the midgut and hindgut of 4th instar *Rhodnius prolixus* nymphs that were uninfected (controls) or infected with *Trypanosoma rangeli*, 12 hours after ecdysis. Shown are control (A, C, E, G) and infected (B, D, F, H) internal morphology from V1 (A, B), V2 (C, D), V3 (E, F), V4 (G, H). Note that V1 of the infected insects are apparently thicker and appear to have more basophilic granules than the control. V1 = ventricle 1, anterior midgut; V2 = ventricle 2, posterior midgut; V3 = ventricle 3, posterior midgut; V4 = ventricle 4, hindgut. Scale= 20 µm. Slides were photographed under a light microscope (40×).

4. Discussion

Insects infected by pathogenic microorganisms can show high mortality during moulting, but this phenomenon has not yet been fully documented and underlying mechanisms have not been investigated. Using the triatomine *Rhodnius prolixus* and the protozoan parasite *Trypanosoma rangeli* as a model system, we investigated development of the new cuticle and ecdysone levels leading up to moult as potential factors to explain this high mortality. Considering the possibility that nutrient depletion may have a role in moulting developmental time, we also examined glycogen content in the fat body, in addition to midgut and hindgut morphology.

Infected insects presented a thinner cuticle compared to that of uninfected insects on the same day of development (except for the tergite and thorax). Ecdysone levels in infected insects were lower than in control insects over time, which can impact insect development and hence survival. Parsimony would suggest that the cuticle is thinner because of delayed cuticle deposition. It also is parsimonious to suppose that the delayed cuticle deposition causes the later moulting of infected insects. This in turn may be related to the low glycogen content of the fat body of infected insects. Considering that nutrition may have a role in cuticle development and formation, we examined the midgut and hindgut epithelium of infected insects but found no cellular pathology. That said, the midgut epithelium of infected insects did appear to be thicker than that of uninfected insects.

The deposition of a new cuticle depends on the levels of ecdysone in the hemolymph. We found lower levels of ecdysone in infected insects compared to control. Low levels of ecdysone over time can affect the development time of the insect, since ecdysone is the hormone that is directly involved in the moulting process. Once abdominal distention has occurred, a signal is sent to the brain that stimulates the synthesis and release of the prothoracicotropic hormone (PTTH). This hormone stimulates the prothoracic gland to synthesize ecdysone. This molecule is released into the haemolymph, being converted into 20-hydroxyecdysone. It activates the cells of the epidermis favoring mitosis for the formation of the new cuticle, and future moult (Gullan and Cranston, 2014). So, if ecdysone levels are low, all this process will last more time to occur. In addition, ecdysone can influence basic patterns of insect physiology as the immune system, what indirectly impacts insect survival. For instance, when the moult of *Rhodnius* sp. infected with bacteria is inhibited in some way, the immune system of these insects is weakened. But when applying ecdysone, the immune system is recovered, and the survival,

which was previously low, now increases (see Azambuja et al. 2017). Likewise, we believe that our mortality results may be impacted by ecdysone levels. Therefore, there seems to be an interaction between ecdysone molecule and the immune system - that could impact insect mortality, but the metabolic route of this interaction is not yet understood.

Interestingly, parasitoid wasps can cause an increase in ecdysone levels in their spider hosts, with a putative role in web construction that would be adaptive for the wasps (Kloss, et al., 2017). However, in our case, parasitized insects showed low levels of ecdysone, which impacts the beginning of the moulting process. Would this outcome be physiological/behavioural manipulation or host defence? We suggest that the changes in ecdysone levels are the result of a manipulation of the host by the parasite, because the ecdysone levels found are low and, as we said before, ecdysone also participates in the immune system. If our results of ecdysone levels were an insect defence, ecdysone levels should be high rather than low.

An important factor that directly influences insect growth and development, and hence cuticle production is nutrient availability (Chapman, 2013). We see that there is more glycogen in infected insects than uninfected. It is probable that infected insects have less glycogen stock because of parasite sequestration of this nutrient. For instance, entomopathogenic fungi can potentially use nutrients such as trehalose (the major sugar used during flight; Becher et al., 1996) in the haemolymph of the host insects by direct uptake (Xia, Clarkson and Charnley, 2002). Other results show that fat body as well as haemolymph of infected insects present more lipids than controls (Ferreira, et al., 2010; AAG, unpublished data). Glycogen decreases as well as lipid increases in parasite-infected insects can indirectly impact adequate deposition of cuticle, and so insect moult. That is because carbohydrates (as glycogen) and lipids are necessary for insect important biological process. Moreover, fly larvae that are infected with a parasitoid has a breakdown of the glycogen molecule, increasing the level of glucose in the haemolymph. That helps the insect to better fight the infection (Ponton, et al. 2019). Thus, maybe there is a consumption of glycogen in pathogen-infected insects as a response to help the insect against the infection.

Moreover, nutrient availability can be impacted by pathogenic microorganisms. For instance, the concentration of some amino acids in the haemolymph is decreased in the presence of *T. rangeli* and this amino acid reduction interferes with the insect's moult (Ormerod, 1967). The bacterium *Nocardia rhodnii*, found in the esophagus and midgut of *R. prolixus*, is essential for its normal growth (Harington, 1960). These symbionts provide folic and pantothenic acids,

pyridoxine, thiamine, and probably nicotinic acid and riboflavin to the insect; these nutrients can be required for normal development and ecdysis (Lake and Friend, 1968). Moreover, when *R. prolixus* is infected with *T. rangeli*, the parasite inhibits *N. rhodnii*, resulting in a vitamin deficiency that can lead to metabolic defects, and thus increase moulting defects and death (Watkins, 1971). It has been suggested that the amino acid tyrosine (Tyr) produced in significant quantities by symbiotic bacteria during insect development is also involved in insect cuticle formation (Anbutsua et al., 2017). Thus, if an insect is infected with a pathogenic microorganism that impacts this symbiont, it can hence impact nutrient availability.

Another way cuticle production may be affected is related to gut damage. If the gut is damaged, the insect may not absorb adequate nutrient levels (Watkins, 1971). We hypothesize that the parasite causes nutritional deficiency leading to a delay in cuticle formation. This leads to a lengthened moult period, and possibly impacts on ecdysis as it seems that the more time it takes for ecdysis to occurs, the greater the chances of death. However, according to our histological analysis of the anterior and posterior midgut, and hindgut, we did not detect clear differences between the epithelium of control and infected treatments, indicating that nutrient absorption by the intestine seems not to be affected by parasites. However, infected insects appear to have a thicker midgut epithelium and more basophilic granules in the cells than controls. That should be studied in more detail for future inferences.

Infection with *T. rangeli* delays moult in *R. prolixus* (Chapter 2), and this result corroborates to other studies that also reported a delayed moult in infected organisms. For instance, *T. cruzi* infection delayed moulting of of *R. prolixus* 2nd instar nymphs, by more than 10 days (Elliot et al., 2015). Also, *Metarhizium* infection delayed moulting of *Schistocerca gregaria* a few days compared with controls, in suboptimal and optimal thermal environments (Elliot et al., 2002). We suggest cuticular thickness (which is directly related to cuticle development) may explain this lengthened moult. Besides our results of cuticular thickness, we noted late formation of new cuticle when dissecting uninfected and infected insects at the same day of development, where the new cuticle was being formed in uninfected insects while it was not present yet in infected insects (see supplementary figure – Fig. S8). We suggest glycogen deficiency is a possible factor to explain developmental delays in moulting since this nutrient possibly impact cuticle formation. Additionally, it may be that delaying moult is adaptive for the parasites, because it delays the insect's next blood-meal (that will occur only once the insect has moulted). It is suggested that this would allow the parasites time to develop and reach the insect salivary gland (considering the *T. rangeli* development cycle), favoring parasite

transmission (Rodrigues et al. 2016). For fungal infection of locusts, something similar could occur, with a delay in moulting favoring pathogen infections. However, it is interesting to highlight that if the insect is under risk of death because of a fungus infection, it moult erlier, and the pathogen might be shed with the moult before penetrating into the host (Duneau and Ebert, 2012).

Moulting is a complex process that involves hormonal, epidermal and cuticular changes, which can be impacted under pathogenic infection circumstances, causing insect death. We suggest this death may be related to ecdysone low levels. Although we did not show a direct relationship between cuticle thickness and insect death during moulting, cuticle thickness is related to the long time taken for infected insects to moult. Such thickness seems to be related to the amount of glycogen in the fat body of the insect. It would be interesting in futures studies to examine effects of infection on amino acid or protein levels leading up to moult, given the cuticle is largely formed from proteins. It would also be interesting to determine if this phenomenon occurs in other host-pathogen systems. Our results provide a mechanistic framework to help understand insect-pathogen interactions related to insect moult physiology. Also, there may be aspects of cuticle formation and how pathogens can impact it that may have led to adaptations in the pathogens to interfere with or manipulate the insect's moult so as to increase its own fitness. This can occur by weakening the host in a way that improves the ability of the pathogen to colonize it. That leads to a weaker cuticle being formed which may facilitate the emergence of the pathogen (as with entomopathogenic fungi that must pass through the cuticle to emerge and sporulate).

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Supplementary material

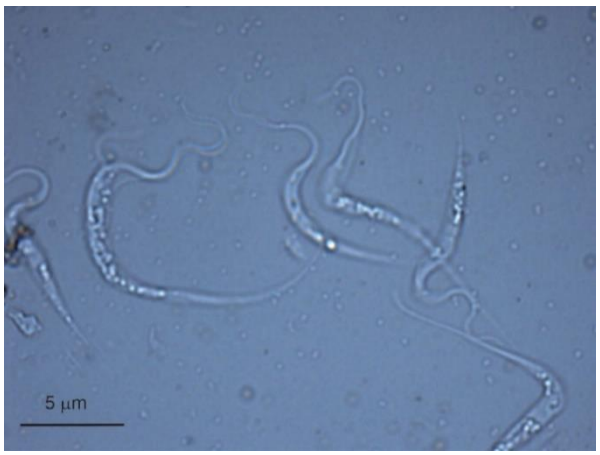

Fig. S1. Visual confirmation of haemolymph infection of 4th instar *Rhodnius prolixus* nymphs infected with epimastigotes stages of *Trypanosoma rangeli*. Samples were examined under a light microscope (100×) to confirm insect parasitemia.

Table S1. Mean ± SE (different letters represent statistical differences at alpha 5%) of cuticular thickness analyses (additionally to Fig. 1). Data are for cuticle sections of abdominal sternites, abdominaltergites and thoraces of 4th instar *Rhodnius prolixus* nymphs that were uninfected (controls) or infected with *Trypanosoma rangeli*, before and after ecdysis. Days are post-feeding, given feeding is usually followed by ecdysis ca. 15 days later. and from infected insects at day 19 (I), still prior to the parasite-delayed ecdysis, and at day 42 (J), after ecdysis.

Cuticular fragment	Treatment	Mean ± SE (µm)		
		Day 9	Day 11	Day 13
Sternite	Control	17.21 ± 1.26a	15.85 ± 1.15a	16.96 ± 1.12a
	Infected	12.06 ± 0.91b	7.55 ± 0.81b	11.49 ± 0.58b
Tergite	Control	19.59 ± 0.15a	20.66 ± 3.64a	18.56 ± 1.97a
	Infected	17.43 ± 9.27b	9.49 ± 2.53a	13.75 ± 2.10b
Thorax	Control	14.75 ± 0.25a	13.49 ± 1.51a	16.61 ± 3.72a
	Infected	7.03 ± 1.46b	17.71 ± 1.23a	11.42 ± 1.21b

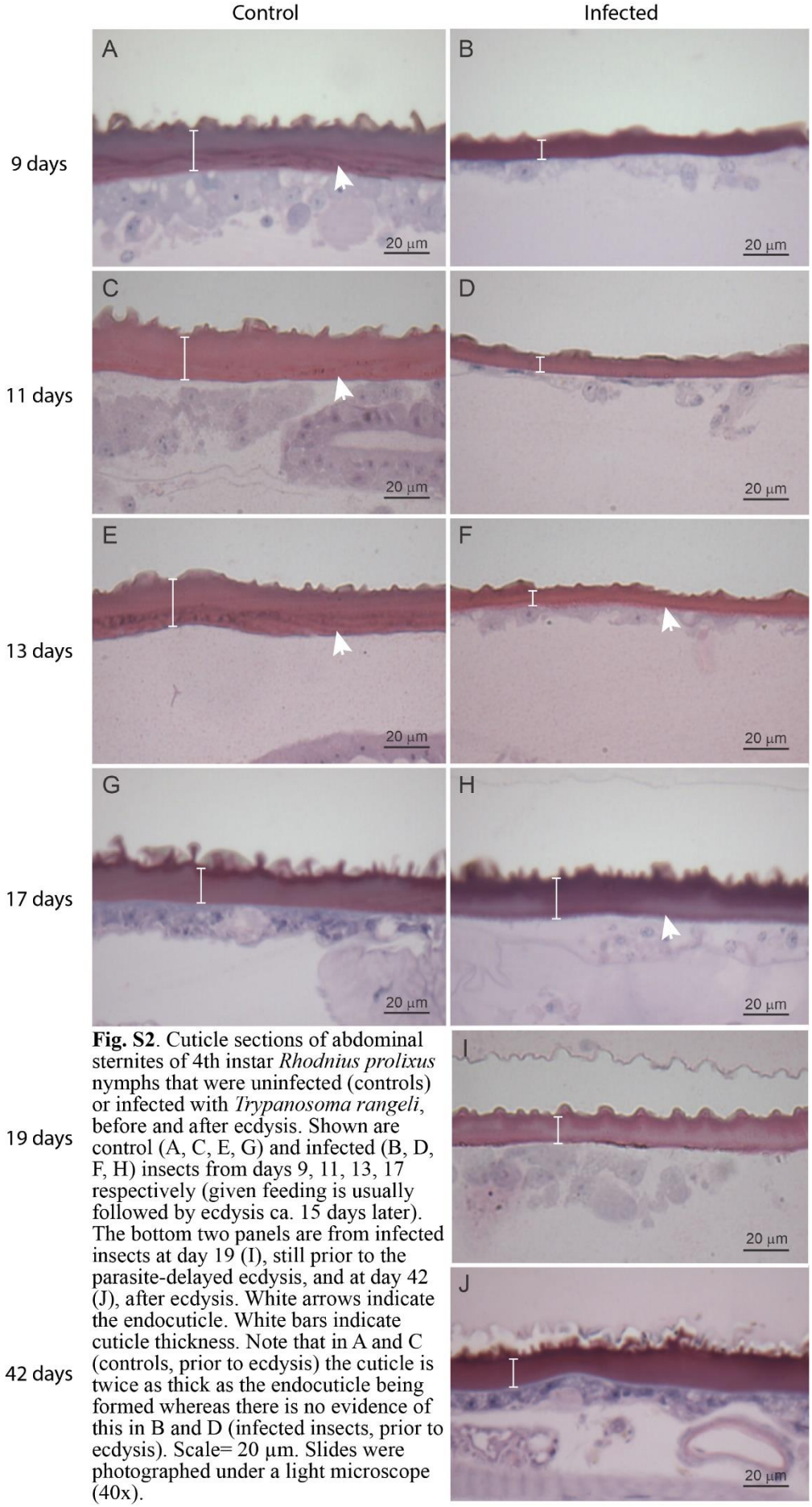

Fig. S2. Cuticle sections of abdominal sternites of 4th instar *Rhodnius prolixus* nymphs that were uninfected (controls) or infected with *Trypanosoma rangeli*, before and after ecdysis. Shown are control (A, C, E, G) and infected (B, D, F, H) insects from days 9, 11, 13, 17 respectively (given feeding is usually followed by ecdysis ca. 15 days later). The bottom two panels are from infected insects at day 19 (I), still prior to the parasite-delayed ecdysis, and at day 42 (J), after ecdysis. White arrows indicate the endocuticle. White bars indicate cuticle thickness. Note that in A and C (controls, prior to ecdysis) the cuticle is twice as thick as the endocuticle being formed whereas there is no evidence of this in B and D (infected insects, prior to ecdysis). Scale= 20 µm. Slides were photographed under a light microscope (40x).

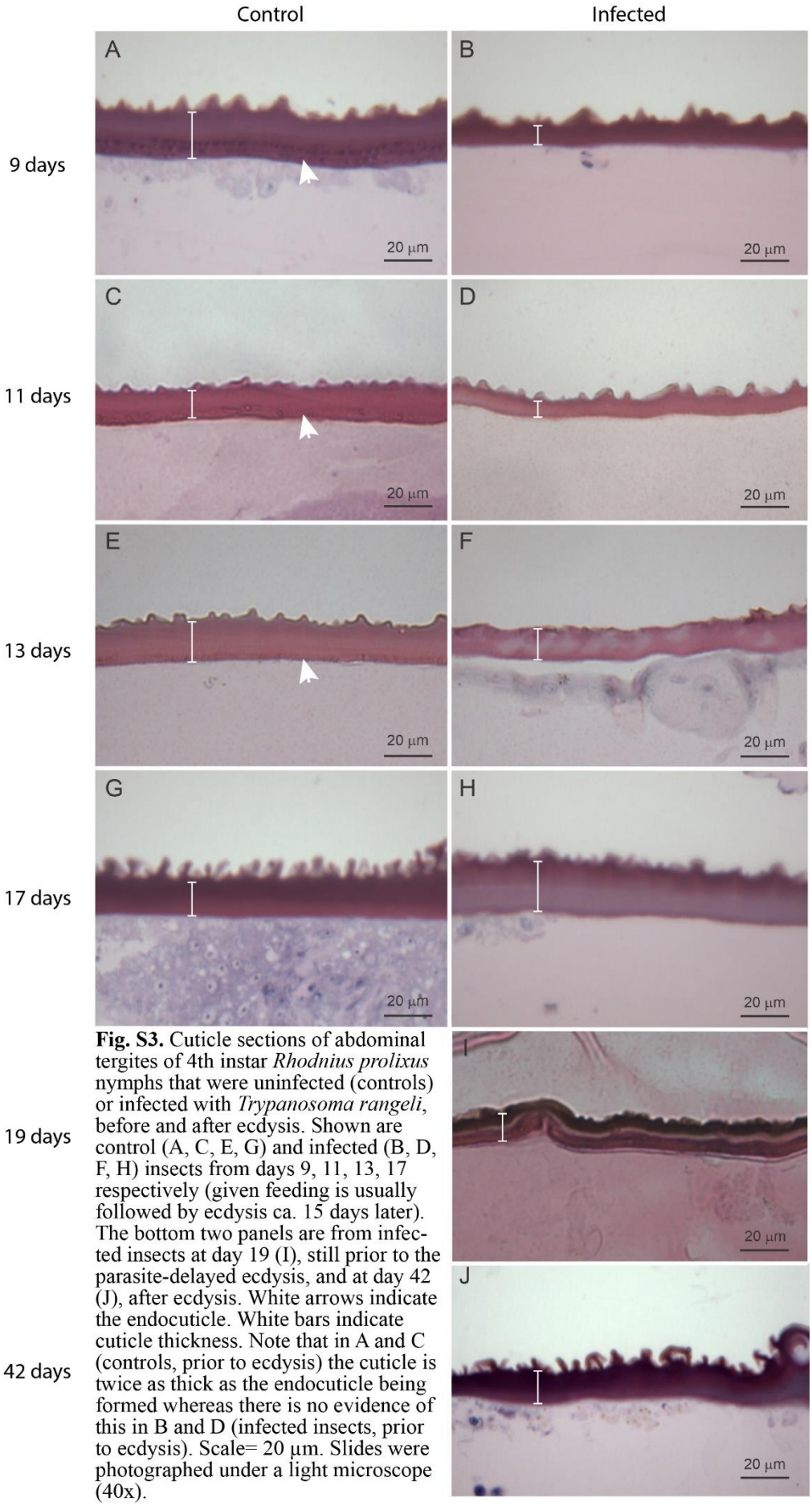

Fig. S3. Cuticle sections of abdominal tergites of 4th instar *Rhodnius prolixus* nymphs that were uninfected (controls) or infected with *Trypanosoma rangeli*, before and after ecdysis. Shown are control (A, C, E, G) and infected (B, D, F, H) insects from days 9, 11, 13, 17 respectively (given feeding is usually followed by ecdysis ca. 15 days later). The bottom two panels are from infected insects at day 19 (I), still prior to the parasite-delayed ecdysis, and at day 42 (J), after ecdysis. White arrows indicate the endocuticle. White bars indicate cuticle thickness. Note that in A and C (controls, prior to ecdysis) the cuticle is twice as thick as the endocuticle being formed whereas there is no evidence of this in B and D (infected insects, prior to ecdysis). Scale= 20 µm. Slides were photographed under a light microscope (40x).

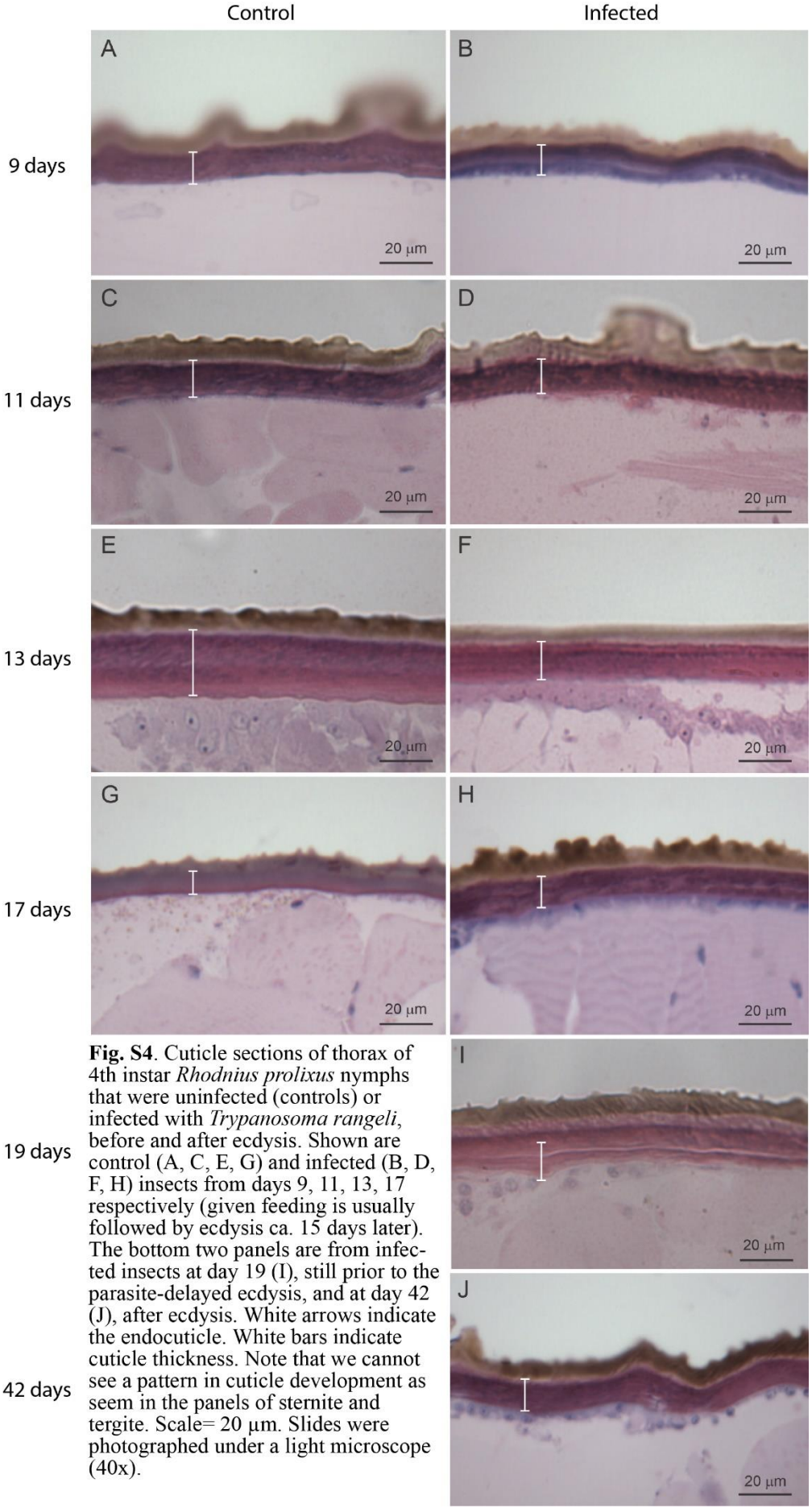

Fig. S4. Cuticle sections of thorax of 4th instar *Rhodnius prolixus* nymphs that were uninfected (controls) or infected with *Trypanosoma rangeli*, before and after ecdysis. Shown are control (A, C, E, G) and infected (B, D, F, H) insects from days 9, 11, 13, 17 respectively (given feeding is usually followed by ecdysis ca. 15 days later). The bottom two panels are from infected insects at day 19 (I), still prior to the parasite-delayed ecdysis, and at day 42 (J), after ecdysis. White arrows indicate the endocuticle. White bars indicate cuticle thickness. Note that we cannot see a pattern in cuticle development as seem in the panels of sternite and tergite. Scale= 20 µm. Slides were photographed under a light microscope (40x).

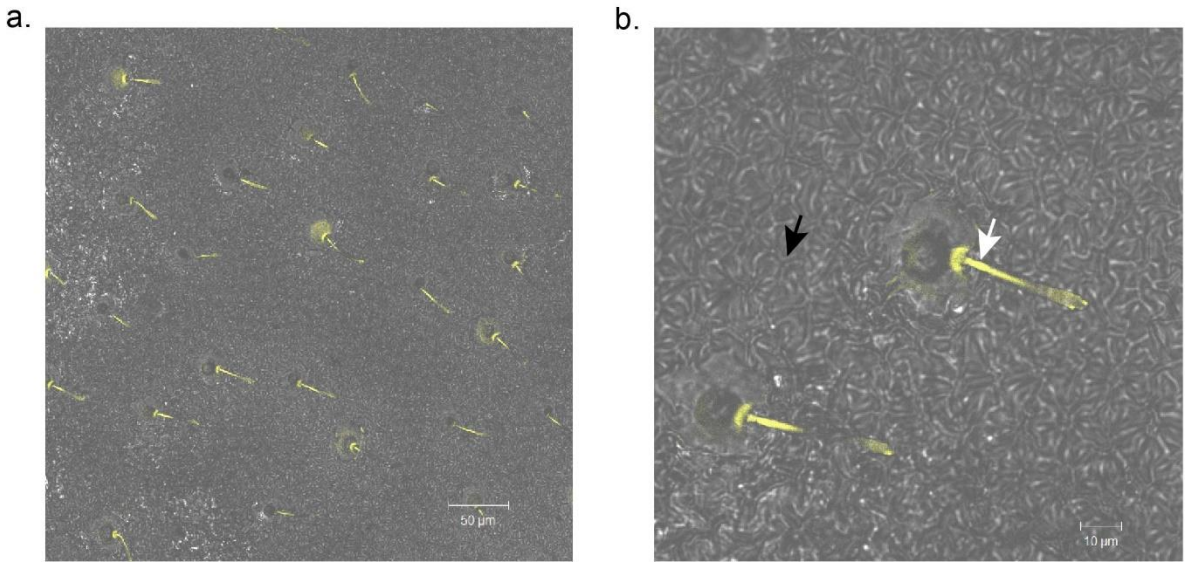

Fig. S5. Outer surface of the cuticle of 4th instar *Rhodnius prolixus* nymphs that were not infected with *Trypanosoma rangeli*) (control). We observed under a microscope that control and infected insects has similar outer surface cuticle. Both have external protuberances that look like small stars in the cuticle outer surface. Scale= 10 µm (a) and 50 µm (b). Slides were photographed under a Confocal Scanning Laser Microscope Zeiss, LSM510 META (20×) – from Núcleo de Microscopia e Microanálise, Universidade Federal de Viçosa (UFV). Black arrow indicates the surface structure of the cuticle. White arrow indicates bristle that fluoresces.

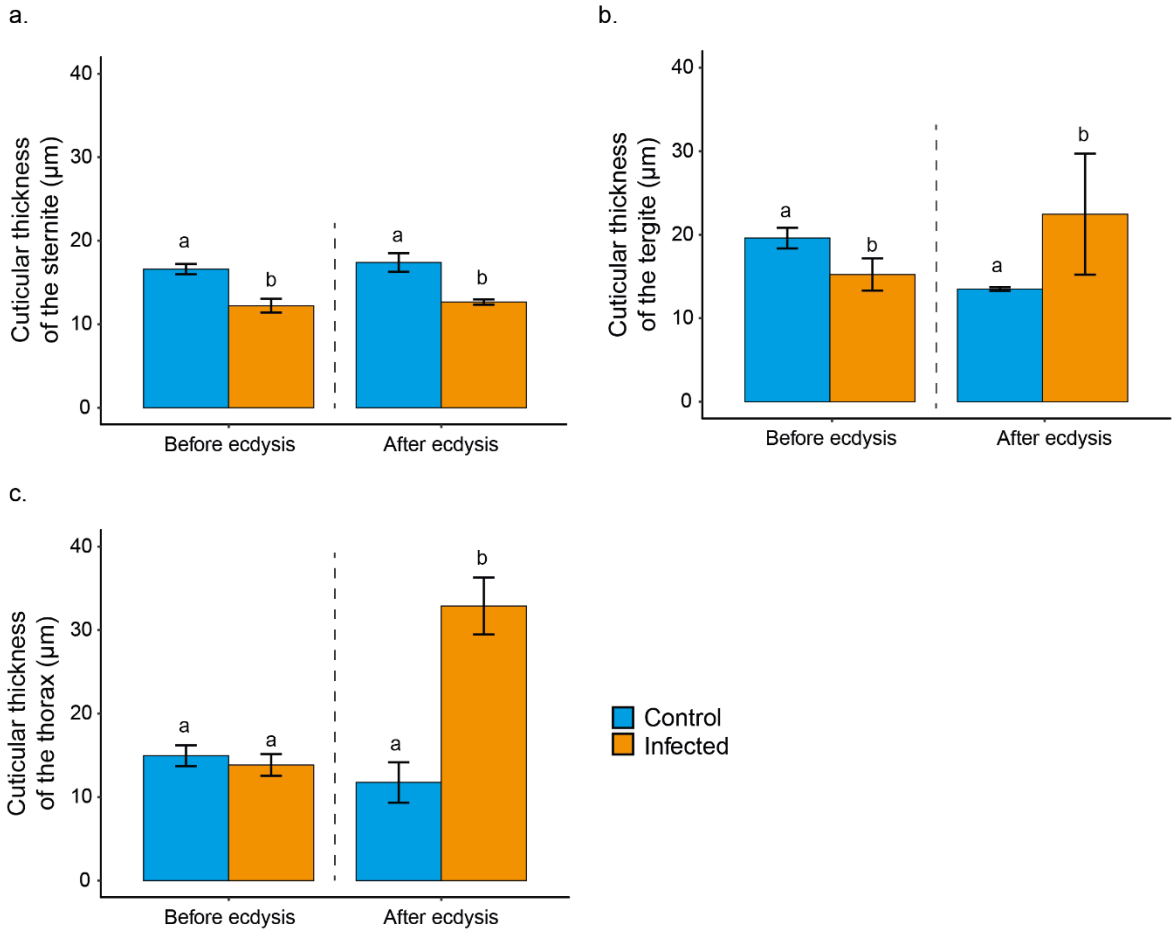

Fig. S6. Cuticular thickness (µm) of sections of sternite (a), tergite (b) and thorax (c) of 4th instar *Rhodnius prolixus* nymphs that were uninfected (control) or infected with *Trypanosoma rangeli*, measured over time. We examined cuticles of control and infected insects before (from days 9, 11 and 13 – given feeding is usually followed by ecdysis ca. 15 days later; also, we measured cuticle of infected insects at day 17 and 19) and 24 hours after ecdysis to 5th instar (days 17 – control, and 42 – infected). Sternite: $F_{(1,26)}$=19.83, P< 0·01; tergite: $F_{(1,26)}$=4.49, P< 0·05; thorax: $F_{(1,25)}$=27.78, P<0·01.

Fig. S7. External structure of the midgut and hindgut of uninfected 4th instar *Rhodnius prolixus* nymphs, 13 days after feeding until repletion. SG = Salivary gland, V1 = ventricle 1, anterior midgut; V2 = ventricle 2, posterior midgut; V3 = ventricle 3, posterior midgut; V4 = ventricle 4, hindgut; MT= malpighian tubules. Scale: 1mm. The samples were photographed under a stereo microscope (Leica), through a camera attached to the eyepiece.

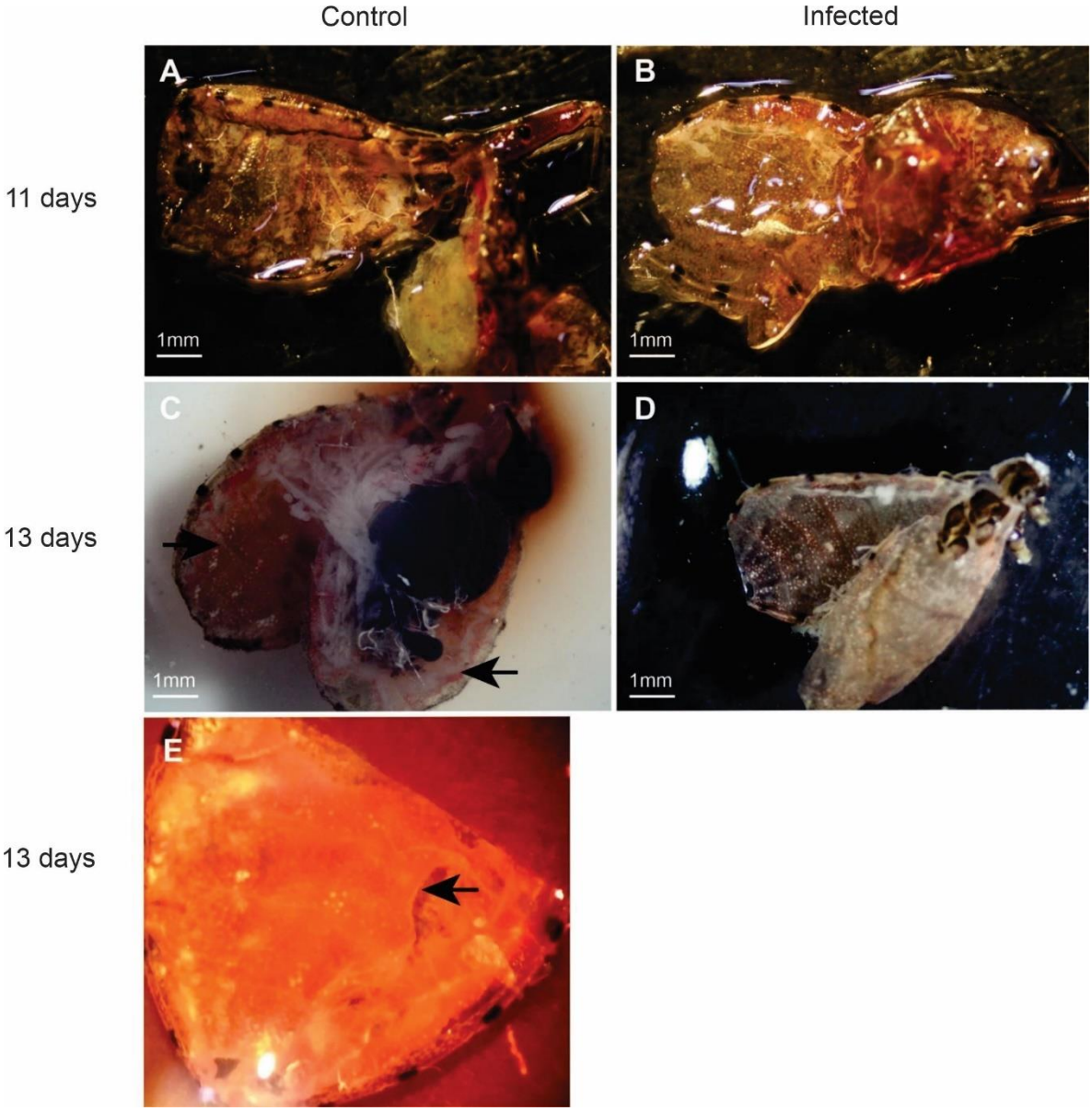

Fig. S8: Cuticle of 4th instar *Rhodnius prolixus* nymphs that were uninfected (controls) or infected with *Trypanosoma rangeli*, before ecdysis. Control (A, C) and infected (B, D) insects from days 11 and 13 after feeding to repletion (given feeding is usually followed by ecdysis ca. 15 days later). Note that we cannot see a new cuticle being formed in images A and B. However, a thin red cuticle is present in control insects from day 13 (shown by a black arrow) (C, E), but we cannot see this in infected insects on the same day (D). Black arrows indicate new cuticle (which is naturally reddish) being formed. Scale are given for images A, B, C and D (1mm). The samples were photographed under a stereo microscope (Leica), through a camera attached to the eyepiece.

FINAL CONSIDERATIONS

Moulting is a complex process, and its success is important for insect growth. When moulting does not occur perfectly, it can cause leg and/or wing deformations, absence of ecdysis and mortality (e.g., Rodrigues et al. 2016). As parasites can alter an insect's physiology, it is important to understand how moulting is affected by these organisms.

As shown in Chapter One, there are four groups of microorganisms (protozoans, fungi, virus) and parasitoids that cause similar effects on moulting. These are delayed moulting, lack of moulting, defective moulting and increased mortality during moulting. Finding these commonalities across such a diverse range of parasites suggests that the underlying mechanisms may be common across these host-parasite interactions and are likely due to disruption of processes, whether physiological, ontogenic or behavioural, in the host.

In Chapter Two, we consider the hypothesis that insect behaviour during ecdysis is altered due to pathogen infection. We quantified mortality in infected insects, and we suggest that the high mortality during moulting in infected insects is influenced by the increased time spent to complete ecdysis. The reason why infected insects take longer to complete ecdysis remain to be understood, and we suggest it is related to air swallowing by the insect. It might be interesting to verify tracheal morphology to look for possible impacts of parasite infection on air swallowing.

In Chapter Three, another (non-exclusive) hypothesis is that negative effects of parasites on insect moulting occur through impacts on development of the cuticle and alterations in levels of ecdysone. Accordingly, we found that the cuticle of infected insects takes longer to be deposited and is probably responsible for the prolonged time to moult. This may be related to the amount of glycogen in the fat body. Related to ecdysone, we found lower levels of this molecule – the hormone that is directly involved in insect moulting – from infected insects compared to control insects, impacting insect development and survival. In addition, ecdysone can also influence insect survival through alterations in the insect immune system (Azambuja et al. 2017).

Considering the moulting delay in *Trypanosoma*-infected insects, it may be adaptive because parasite infection delays the insect's next blood meal, as this only occurs after the insect has moulted. This delay could allow the parasite to complete its cycle. Considering fungi infecting insects, if the inability to walk or fly means that an insect falls to the ground, a fungus can be found in the soil and be better able to sporulate and infect new hosts.

In Chapter 3, we also investigated midgut and hindgut morphology as possible factors increasing the development time of moulting as results of infection. We found that infected insects do not have damage in the midgut epithelial cells, but the epithelium is apparently thicker than the control. However, more studies are needed to confirm effects of parasites on intestinal epithelium. We also suggest futures studies to examine effects of infection on protein levels leading up to moult. It would be also interesting to determine if this phenomenon occurs in other infection systems.

Our results allow us to understand in more depth the effect of insect infections at behavioural, morphological and hormonal levels. It is also important to highlight the question of how this might be adaptive for the parasite would be if the insect is dying. The infection generated negative effects on glycogen availability and ecdysone levels, for example, but other related variables may not be affected. This may be contributing to a more positive than negative balance in relation to insect growth. Furthermore, effects of infection on moulting may be commonplace, but many studies end up not reporting such effects even when they occur.