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Benefits and costs of immune memory in *Rhodnius prolixus* against *Trypanosoma cruzi*

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ABSTRACT

There is increasing evidence supporting the immune memory in invertebrates, but the studies are relatively neglected in insect vectors other than mosquitoes. Therefore, we tested two hypotheses: 1) *Rhodnius prolixus* insects possess immune memory against *Trypanosoma cruzi*, and 2) their immune memory is costly. The Dm28c and Y strains of *T. cruzi* were used, the former being more infective than the latter. On the one hand, the triatomines subjected to dual challenges with the Dm28c strain did not show significant differences in survival than those of the heterologous challenge groups control-Dm28c and Y-Dm28c. On the other hand, the insects survived longer after a dual Y–Y challenge than after the corresponding heterologous challenge (control-Y). The Y–Y, Dm28c-Y, and naïve groups showed similar survival. There was more prolonged survival following the Y–Y versus Dm28c-Dm28c dual challenge. The Dm28c-Dm28c group exhibited moulting sooner than the control-Dm28c or naïve group. In contrast, there were no differences in the probability of moulting between the Y–Y and naïve groups. The results suggest that triatomines have immune memory against the Y but not the Dm28c strain. Further investigation on triatomine and *T. cruzi* interaction is needed to determine if infectivity accelerates or delay growth due to innate immune memory.

1. Introduction

Innate immune memory, long thought to exist only in vertebrates, has been demonstrated in invertebrates during the last two decades [1]. According to the innate immune memory, dual homologous challenges (similar strains or species) provide specific protection during the second infection, resulting in increased survival [1]. However, despite the apparent benefits of immune memory favoring survival, recent studies have found that memory is costly [2]. For example, organisms that develop immune memory after facing a homologous challenge have delayed growth in comparison with those without immune memory [3]. An excellent way to assess growth in ecdysozoans is the occurrence of ecdysis because ecdysone favors the change of exoskeleton as a result of growth [4]. Therefore, an insect with average growth undergoes moulting sooner than an individual with delayed development (growth arrest).

Even though the reports on innate immune memory have increased in the last ten years in invertebrates, there are no studies in this subject in triatomine insects against the etiological agents of Chagas disease [reviewed in 1,5] and, if such immune memory exists, whether it is costly [5]. Hence, in the current contribution, we tested the hypothesis that the triatomine *Rhodnius prolixus* has immune memory against *T. cruzi*, predicting longer survival for the insects confronted with a dual homologous versus heterologous challenge. Additionally, we propose that immune memory in *R. prolixus* is costly, predicting that organisms with immune memory (dual homologous challenge) would have delayed moulting when compared with those without memory (dual heterologous challenge).

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2. Methods

2.1. Insects and parasites

The experiments were carried out at *Laboratório de Bioquímica e Fisiologia de Insetos* (LABFISI) of *Instituto Oswaldo Cruz* (IOC) at the *Fundação Oswaldo Cruz* (Fiocruz) in Rio de Janeiro, Brazil. *R. prolixus* (n = 378) were maintained at 26–27 °C and 55–60% relative humidity [6]. The triatomines were fed artificially with rabbit blood to an artificial apparatus either uninfected [7] or infected with the Dm28c or Y strains of *T. cruzi*. Both strains were maintained with brain heart infusion (BHI) medium complemented with 10% fetal bovine serum, hemin, and folic acid [8]. Rabbit blood was provided by the *Instituto de Ciência e Tecnologia em Biomodelos* (ICTB/Fiocruz), which maintains and breeds animals following the Ethical Principles in Animal Experimentation. Blood collection was licensed and approved by *Comissão de Ética no Uso de Animais* from *Fundação Oswaldo Cruz* (CEUA/Fiocruz) under the protocol number L-019/17.

2.2. Experimental design

Three groups of *R. prolixus* of the fourth instar were established (see sample size in results), each fed with a distinct type of blood: 1) uninfected (the naïve control), 2) containing dead Dm28c *T. cruzi* parasites (Dm28c group), and 3) containing dead Y *T. cruzi* parasites (Y group). Dead parasites were used to activate the immune response without causing an infection. The parasites were collected in the exponential growth phase and were washed three times by centrifugation at 1000*g* in phosphate buffer saline (PBS, Sigma) at 4 °C for 20 min. They were fixed with 0.1% glutaraldehyde and washed with PBS [9]. These parasites were added to rabbit blood to feed the kissing bugs. Following the first feeding, all three groups of insects were monitored for survival and moulting until reaching the fifth instar, at which point they were given the second feeding with live parasites.

Surviving insects from all three groups after the first food received a second feeding (see sample size in results) but involving live parasites (not treated with glutaraldehyde). This experimental procedure using dead enemies allows the insect's immune system to recognize the parasite after the first encounter, controlling for potentially confounding factors like parasite virulence and differential survival by the host. Under the immune memory rationale, this activation favors the elimination of live parasites after the second exposure [see refs. 2, [10]. Insects were manipulated in the same way to avoid any skew.

To test immune memory against Dm28c, we established four groups in a full factorial design [see 2, 10]. One group was twice fed with uninfected blood (naïve), a heterologous group was first fed with uninfected blood and then with blood containing Dm28c parasites (control-Dm28c group), another heterologous group was fed with blood containing Y parasites and then with blood containing Dm28c parasites (Y-Dm28c), and finally, a homologous group was fed twice with dead Dm28c parasites and then were fed with live Dm28c parasites (Dm28c-Dm28c group). We established four groups to test immune memory against Y: naïve, control-Y, Dm28c-Y, and Y–Y. The infected blood contained the same doses of 1×10^6 parasites/mL, but in the first blood, they were dead and lived in the second fed. After the second feeding, all triatomines were monitored for survival (insects were gently touched with entomological forceps to ensure that they were dead, meaning that they did not react to the touch) and moulting until adulthood (for about 20–35 days; see results). These tests were blind in the sense that the person that checked survival and moulting did not know the groups' identity.

Finally, we tested whether strains Y and Dm28c differed in infectiveness to explain potential differences in immune memory due to strains. In short, we took the intestine of each bed bug to macerate it in 1 mL Eppendorf tubes that contained 1 mL of PBS. From this mash, we took 1 μL to be deposited in a Neubauer chamber to count the parasites under the microscope. This technique is widely used to count parasites [11].

3. Results and discussion

There was no significant difference in survival ($X^2 = 0.65$; d.f. = 2; p = 0.72) between those insects feed with uninfected blood (naïve; n = 176), the dead Y strain (n = 109) or the dead Dm28c strain (n = 93). In addition, there were no significant differences in moulting in this same 3 groups ($X^2 = 2.16$; d.f. = 2; p = 0.34). This is important because suggests that the activation of the immune response alone, does not affect survival or development.

No significant differences were found in the survival between the heterologous control-Dm28c (n = 39), Y-Dm28c (n = 33) or the dual homologous challenge with the Dm28c strain (n = 41; $X^2 = 1.96$; d.f. = 2; p = 0.37). However, insects in the heterologous group control-Y (n = 36) died sooner than those in the Dm28c-Y (n = 44), Y–Y (n = 31) or naïve groups (n = 70; $X^2 = 13.48$; d.f. = 3; p = 0.004; Fig. 1). This is that the activation of immune response with Dm28c or Y protects against the live Y strain, but any previous protection favors the insect's susceptibility against the live Y strain. This is the first evidence of innate immune memory in triatomines.

It has been demonstrated that the invertebrate's immune memory may occur at a strain level [12,13], but here, we did not find strain-specificity using the Y and Dm28c strains. Further research should test whether there is a strain-specific immune memory against other strains of *T. cruzi* or strains from another parasite species such as *T. rangeli*. Specially, given the host-parasite co-evolution and its geographical diversity [14]; see also 5]. Increasing studies in other species, taking into account the phylogeny, may establish testable hypotheses about the evolution (and possibly, co-evolution) of immune memory according to parasites (a neglected topic in innate immune memory).

Still, despite the elucidation of the specificity by the triatomines immune response, another key result was the immune memory against Y but not Dm28c. The innate immune memory is influenced by the species and virulence of the pathogen [15]. *T. cruzi* kills about 38% of the kissing bugs that feed on infected blood [16], and the Dm28c strain is more infective than the Y strain [17]. Indeed, here we found that insects confronting the Y strain were less infected (0.04 ± 0.04 parasites per insect; n = 24) than those confronting Dm28c (404800.0 ± 102744.2 parasites per insect; n = 25; t = −3.85, d.f. = 47; p < 0.0001). Although no significant differences in survival were detected between the groups with heterologous challenges control-Y *versus* control-Dm28c ($X^2 = 1.44$; d.f. = 2; p = 0.23), a significant difference did indeed exist between the groups with the homologous challenges: The Y–Y group survived longer than the Dm28c-Dm28c group ($X^2 = 8.0$; d.f. = 2; p = 0.005). This supports the hypothesis that innate memory may not occur against some parasite strains or species due to virulence [2]. Hence,

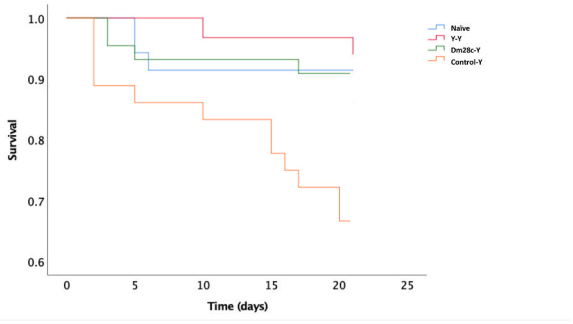

Fig. 1. Insects of the homologous challenges Y–Y (memory), naïve and Dm28c-Y survived more days than the control group (control-Y). There were no significant differences in survival between Y–Y, naïve, and Dm28c-Y.

innate immune memory should not be rejected before putting it to test by using distinct pathogen or parasite strains or species [2].

Regarding development and ecdysis, diapause is considered an adaptive strategy under stressful conditions (the bet-hedging hypothesis) [18]. In *Triatoma infestans*, the nymphs exhibit delayed growth in response to the insecticide deltamethrin [19] and viral infections [20]. In triatomines, including *R. prolixus*, a delay in development could respond to environmental challenges [21,22] if the delay allows the conditions to improve the adults' performance [21]. The insects of the Dm28c-Dm28c group had a greater probability of moulting than the control-Dm28c ($X^2 = 3.64$; d.f. = 2; p = 0.05) or naïve group ($X^2 = 19.39$; d.f. = 2; p < 0.0001). Additionally, the control-Dm28c group underwent ecdysis sooner than the naïve group ($X^2 = 6.3$; d.f. = 2; p = 0.01; Fig. 2). These results contradict the idea that organisms delay their growth when faced with adverse conditions. However, insects are also known to accelerate their development to escape infections [23]. In ecdysozoans, this phenomenon has been reported against fungi and bacteria [23,24]. Accordingly, here no significant differences were found in the probability of moulting between the Y–Y, control-Y, and the naïve groups ($X^2 = 5.38$; d.f. = 2; p = 0.07). We hypothesize that the triatomines confronted with Dm28c could have opted for accelerated growth to convert themselves into adults, while the insects faced with the less infective strain (Y) may have opted for the strategy innate immune memory. In the first case, it would be interesting to know if this strategy is according to terminal investment, which means that insects may accelerate their development to get a reproductive benefit at a cost in later survival [25]. It is necessary to carry out a study in the long term to know the cost/benefit due to developmental acceleration after dual homologous challenges compared to heterologous challenges and according to parasite virulence and infectivity. In the second case, the possible cost of the investment in innate immune memory should be considered. Nevertheless, no delay in growth was observed for the Y–Y versus the naïve group. Both hypotheses grant further research because this is the first time the phenomenon is proposed in invertebrates under the topic of innate immune memory.

The developmental plasticity of kissing bugs is a key question in triatomines evolution and live-history evolution [26]. We propose that this is also a good subject for further study to ascertain whether infections cause a delay or acceleration of growth and whether innate immune memory imposes a cost on development. The current hypothesis in the literature of immune memory is that developmental changes reflect a cost of immune memory. However, we propose that such changes be plastic to combat infections and opt for rapid development to reach the adult stage. Although both ideas incorporate trade-offs, the former suggests a cost and the latter a strategy to optimize reproduction. It will be crucial to investigate a delay or acceleration of development in the short, medium, and long-run to discern between these hypotheses.

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CRediT authorship contribution statement

S.P. Carmona-Peña: Writing – original draft, Investigation, Formal analysis. **J.C. Vázquez-Chagoyán:** Writing – review & editing, Conceptualization. **D.P. Castro:** Writing – review & editing, Writing – original draft, Conceptualization. **F.A. Genta:** Writing – review & editing, Methodology, Conceptualization. **J. Contreras-Garduño:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Formal analysis, Conceptualization.

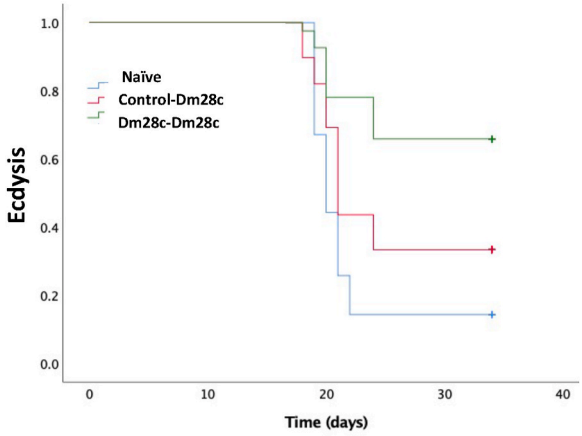

Fig. 2. Insects of the homologous challenges Dm28c-Dm28c (memory) grew faster compared with naïve and the control groups (Control- Dm28c).

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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