University of São Paulo
"Luiz de Queiroz" College of Agriculture

Phenotypic diversity in the biological and behavioral responses of isolines of *Myzus persicae* (Sulzer, 1776) (Hemiptera: Aphididae) to *Diaeretiella rapae* (M'Intosh, 1855) (Hymenoptera: Braconidae)

Mariane Possignolo Gomes

Dissertation presented to obtain the degree of Master in Science. Area: Entomology

Piracicaba
2021

Mariane Possignolo Gomes
Teaching licensed and Bachelor in Biological Sciences

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DEDICATION

To my mother, Vilma, who is an example of a warrior woman that has always been by my **s**ide in the good and bad times, providing encouragement and the support to never let me give up on my dreams.
To my brothers Gabriel and Gabriele, who always believed in my potentials, even when I did not believe myself.
To my fiancé Vilmar, who shared my dreams and never stopped supporting me even in the most difficult times.
To my lab friends, Lilian and Éwerton for all their emotional support, and for believing in me.
I dedicate this work ...

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"Those who pass by us, do not go alone, and do not leave us alone; they leave a bit of themselves, and take a little of us."

(Antoine de Saint-Exupéry)

“To myself I am only a child playing on the beach, while vast oceans of truth lie undiscovered before me.”

Isaac Newton

CONTENTS

Resumo 9

Abstract 10

LIST OF FIGURES 11

LIST OF TABLE 13

1. Introduction 15

REFERENCES 20

2. Variation in isolines of *Myzus persicae* to successful parasitization by *Diaeretiella rapae* and assessment of biological traits of selected lines 27

Abstract 27

2.1. Introduction 27

2.2. Material and methods 30

2.2.1. Establishment and maintenance of aphid isolines 30

2.2.2. Plants 30

2.2.3. Parasitoids 31

2.2.4. Insects taxonomic and molecular characterization 31

2.2.5. Diagnostic-PCR detection of aphids secondary symbionts 33

2.2.6. Assessing the parasitization of *Myzus persicae* isolines by *Diaeretiella rapae* 35

2.2.7. Evaluation of biological parameters of isolines of *Myzus persicae* 35

2.3. Results 36

2.3.1. Insect identification and molecular characterization 36

2.3.2. Aphid symbionts detection 38

2.3.3. Assessing parasitization of *Myzus persicae* isolines by *Diaeretiella rapae* 39

2.3.4. Evaluation of biological parameters of isolines of *Myzus persicae* 39

2.3.4.1. Immature and adult biological parameters 39

2.3.4.2. Fertility life table 43

2.4. Discussion 45

2.5. Conclusions 47

REFERENCES 48

3. Behavioral analysis of the interaction of *Myzus persicae* isolines - *Diaeretiella rapae* 55

Abstract 55

3.1. Introduction 55

3.2. Material and methods 57

3.2.1. Insects 57

3.2.2. Parasitism assays 57

3.3. Results 58

3.3.1. Aphid and parasitoid behavior 58

3.4. Discussion 62

3.5. Conclusions 64

REFERENCES 65

RESUMO

Diversidade fenotípica nas respostas biológicas e comportamentais de isolinhagens de *Myzus persicae* (Sulzer, 1776) (Hemiptera: Aphididae) a *Diaeretiella rapae* (M'Intosh, 1855) (Hymenoptera: Braconidae)

Interações ecológicas são onipresentes e ocorrem entre todos os organismos, macro ou microrganismos. Na relação inseto-inseto, parasitoides são os principais entomofagos e são explorados em programas de controle biológico de diferentes insetos de importância agrícola. A compreensão da interação hospedeiro-parasitoide é essencial para o desenvolvimento de pesquisas aplicadas para a implantação de programas bem-sucedidos de controle biológico em campo. A relação estabelecida entre hospedeiro e parasitoide é resultado de processos coevolutivos, nos quais fenótipos de parasitoides com habilidades de ataque foram selecionados em resposta às adaptações de defesa do hospedeiro. Em contraste, mecanismos de defesa desenvolvidos para driblar estratégias de ataque de parasitoides, envolvendo adaptações comportamentais e fisiológicas foram selecionadas na população hospedeira. A evolução de mecanismos de defesa desenvolvidos pelo hospedeiro pode resultar em custos biológicos, refletidos na fecundidade ou tamanho do inseto, influenciando, assim, a aptidão biológica do hospedeiro. Com o objetivo de identificar a diversidade fenotípica nas respostas biológicas e comportamentais apresentadas por hospedeiros ao ataque por inimigos naturais, investigamos a interação hospedeiro *Myzus persicae* - parasitoide *Diaeretiella rapae* usando isolinhagens de *M. persicae* com diferentes respostas ao ataque do parasitoide. O sucesso do parasitismo observado de *M. persicae* por *D. rapae* variou entre 43% e 76% nas 14 isolinhagens testadas, das quais foram selecionadas três isolinhagens do primeiro (maior parasitismo) e do quarto (menor parasitismo) quartis para avaliação de parâmetros biológicos e comportamentais. Os parâmetros biológicos avaliados demonstraram diferenças significativas entre isolinhagens de *M. persicae* com diferentes respostas ao parasitismo, mas sem a associação uniforme de custo à capacidade de resposta ao parasitismo, para as diferentes isolinhagens estudadas. A associação de *M. persicae* com o simbionte secundário normalmente relatados a pulgões, *Rickettsia*, sugerem que esses organismos podem não afetar diretamente a capacidade do pulgão em responder ao parasitoide *D. rapae*, mas linhagens infectadas apresentaram maior capacidade reprodutiva. *Rickettsia* também demonstrou influenciar o comportamento de defesa das isolinhagens testadas, com as fêmeas de pulgões infectadas apresentando maior frequência de agitação do corpo do que linhagens não-infectadas. Infecção do hospedeiro por *Rickettsia* também interferiu no comportamento de seleção hospedeira dos parasitoides, que atacaram com maior frequência as isolinhagens livres do simbionte secundário, bem como pulgões do grupo de menor parasitismo. A presença de comportamento de defesa, representado por movimentos do corpo mais intensos no grupo de menor parasitismo, pode justificar o maior número de ataques necessários para parasitá-los, enquanto que o menor número de ataques pelo parasitoide em pulgões infectados por *Rickettsia*, mesmo que eles tenham apresentado comportamento de defesa semelhante ao do grupo de menor parasitismo, sugere que esse simbionte pode ter induzido outras alterações nos pulgões que interferiram no processo de seleção hospedeira do parasitoide, indicando a necessidade de estudos fisiológicos para entender os fatores envolvidos no parasitismo observado nas isolinhagens selecionadas de *M. persicae*.

Palavras-chave: Interação hospedeiro-parasitoide; Variação fenotípica; Resposta ao parasitismo; Custos adaptativos

ABSTRACT

Phenotypic diversity in the biological and behavioral responses of isolines of *Myzus persicae* (Sulzer, 1776) (Hemiptera: Aphididae) to *Diaeretiella rapae* (M'Intosh, 1855) (Hymenoptera: Braconidae)

Ecological interactions are ubiquitous and occur between all macro or microorganisms. In insect-insect interactions, parasitoids are the main group of entomophagous that are exploited in biological control programs for many agricultural insect pests. Understanding the host-parasitoid interactions is essential for the development of applied research for the implementation of successful biological control programs in the field. The relationship established between host and parasitoid is the result of coevolutionary processes, in which phenotypes of parasitoids with attacking abilities were selected in response to the host's defense adaptations. In contrast, defense mechanisms developed to circumvent parasitoid attack strategies, involving behavioral and physiological adaptations were selected in the host population. The evolution of defense mechanisms developed by the host can result in biological costs, reflected, for example, in the low fertility or small size of the insect, influencing the biological aptitude of the host. To identify the phenotypic diversity in the biological and behavioral responses presented by hosts to the attack of natural enemies, we investigated the interaction between the host *Myzus persicae* and the parasitoid *Diaeretiella rapae*, using isolates of *M. persicae* with different responses to the attack of the parasitoid. The success of the observed parasitism of *M. persicae* by *D. rapae* ranged from 43% to 76% among 14 tested isolines. Three isolines with parasitism rate in the first (high parasitism) and fourth (low parasitism) quartiles were selected for the evaluation of biological and behavioral parameters. The biological parameters evaluated showed significant differences between lineages of *M. persicae* with different responses to parasitism, but without a uniform association of cost with the aphid capacity to respond to parasitism. The association of *M. persicae* with the secondary symbiont *Rickettsia* demonstrates this symbiont does not interfere directly with the aphid's ability to respond to the parasitoid *D. rapae*, but it had a positive effect in the fecundity of infected isolines. *Rickettsia* infection also influenced the defense behavior of the tested aphid isolines, with infected aphid females showing a higher frequency of body wiggling than the uninfected females. Host infection with *Rickettsia* also interfered in the host selection behavior of *D. rapae*. Parasitoid females attacked more frequently *Rickettsia*-free isolines. Aphids from selected isolines with low parasitism by *D. rapae* were also more attacked than the aphids from isolines with high parasitism. The defensive behavior displayed by wiggling the body more intensively in the group aphids with low parasitism can justify the required larger number of attacks for their successful parasitization. In *Rickettsia*-infected aphids we observed a low number of attacks, although aphids also wiggled their bodies more intensively than uninfected aphids. In this case, we argue that *Rickettsia* induces other physiological changes in the host that it affects the host selection behavior of *D. rapae*, suggesting the need of further physiological studies for a better understanding of the factors involved in the observed parasitization of the selected isolines of *M. persicae*.

Keywords: Host-parasitoid interaction; Phenotypic variation; Response to parasitism; Adaptive costs.

LIST OF FIGURES

Figure 1: Representative illustration of strategy used for amplification of amplicons of the cytochrome oxidase I gene of Diaeretiella rapae and Myzus persicae..... 32

Figure 2. Percentage of isolines identified as Brevicoryne brassicae, Lipaphis pseudobrassicae or Myzus. persicae..... 37

Figure 3. Phylogenetic tree of aphid species using partial sequences of the gene COI analyzed by the maximum-likelihood method. The scale bar indicates substitution by nucleotide position..... 37

Figure 4. Phylogenetic tree of parasitoids species using sequence of gene COI analyzed by the maximum-likelihood method. The scale bar indicates substitution by nucleotide position..... 38

Figure 5. Clone lines of Myzus persicae used in parasitism test with infection by Buchnera aphidicola, Rickettsia and/or Spiroplasma..... 38

Figure 6. Successful parasitization of different isolines of Myzus persicae by Diaeretiella rapae. Red line = 75% percentile; purple line = 25% percentile..... 39

Figure 7. Immature development of Myzus persicae from selected isolines for high (ISO2, ISO3 and ISO4) and low (ISO10, ISO11 and ISO14) parasitization by Diaeretiella rapae. A) Probability of nymphs of M. persicae to reach adulthood. Treatments followed by different letters are statistically different (Cox proportional hazards models, $p < 0.05$). B) Immature development time. Treatments followed by different letters are statistically different (Kruskal-Wallis test. $p < 0.05$). Green bars= indicate the lineages of the group with lower parasitism. Blue bars= represent the isolines with higher parasitism. 40

Figure 8. Daily female survival (%) and relative accumulated fecundity (%)of females of Myzus persicae from selected isolines for high (ISO2, ISO3 and ISO4) and low (ISO10, ISO11 and ISO14) parasitization by Diaeretiella rapae..... 42

Figure 9.Pair correlation plot of fertility life table parameters of selected isolines of Myzus persicae with different responses to parasitization by Diaeretiella rapae..... 44

Figure 10. Cluster heatmap of reproductive life table data. The hierarchical clustering was generated using Euclidean distance and Ward's method. The color scale indicates the degree of correlation (white= low correlation; red= high correlation). 44

Figure 11. Behavioral parameters of Diaeretiella rapae exploiting patches with isolines of Myzus persicae selected from groups with high and low parasitism by D. rapae. A) Average time outside from patch; B) Number of encounters with the host; C) Number of host evaluations; D) Number of cleaning. 60

Figure 12. Average attacks of Diaeretiella rapae parasitoid in isolines of Myzus persicae from groups with high and lower parasitism and Rickettsia-free and Rickettsia-infected. A) Average attacks number by D. rapae in high (blue bars) vs low (dark green bars) and Rickettsia-infected (light green) vs Rickettsia-free (pink bars). * indicates statistical differences ($p < 0.05$). 60

Figure 13. Intensity (n) (A) and duration (s) (B) of body wiggling as a defensive behavior of selected isolines of Myzus persicae for high (blue color - ISO2, ISO3 and ISO4) and low (green color - ISO10, ISO11 and ISO14) parasitism by Diaeretiella rapae. 62

LIST OF TABLE

Table 1. Specific primers and PCR conditions used in diagnostic PCRs for the identification of secondary symbionts associated with isolines of Myzus persicae. 34
Table 2. Fecundity (nymphs/female), tibia size (µm) and longevity (days) of adult females from selected isolines of Myzus persicae for high (ISO2, ISO3 and ISO4) and low (ISO10, ISO11 and ISO14) parasitization by Diaeretiella rapae. 41
Table 3. Life table parameters of selected isolines of Myzus persicae for high (ISO2, ISO3 and ISO4) and low (ISO10, ISO11 and ISO14) parasitization by Diaeretiella rapae. 43
Table 4. Behavioral parameters of Diaeretiella rapae exploiting patches with selected isolines of Myzus persicae with high and low successful rates of parasitism, carrying ($Rick^+$) or not ($Rick^-$) Rickettsia infections. 59
Table 5. Behavioral parameters of Myzus persicae evaluated for isolines classified into higher and lower parasitism groups and Rickettsia-free and Rickettsia-infected. 61

1. INTRODUCTION

Aphids are phytophagous, sap-sucking hemipterans considered to have a great importance for agriculture worldwide due to the damage they cause to several crops, such as cereals, vegetables, and fruits, resulting in great economic losses (Dedryver et al., 2010; Katis et al., 2007). Aphids cause direct damage by sucking the sap of host plants and by secreting toxic saliva, leading to leaf malformation and to host nutritional deficiencies (Blackman & Eastop, 2021; Dedryver et al., 2010; Ellis et al., 1996). Aphids can also cause indirect damage by reducing the host photosynthetic capacity due to the growth of fungi on the honeydew accumulated over the surface of leaves (Blackman & Eastop, 2021; Ellis et al., 1996). But the major indirect damage aphids cause to host plants is due to the vectoring of more than one hundred types of plant viruses, such as the Bean Leafroll Virus (BLRV), Beet Yellow Net Virus (BYNV), Pea Enation Mosaic Virus (PEMV), Barley yellow dwarf virus (BYDV) and the Potato Leafroll Virus (PLRV), which delay host plant development by interfering with the growth, flowering, and fruiting of the host plant (Blackman & Eastop, 2021; Dixon, 1981; Jarosová et al., 2016; Ortiz et al., 2005). In tropical countries, most aphid species, such as *Myzus persicae*, reproduce exclusively by ameiotic (apomictic) parthenogenesis, producing genetically identical progenies. This reproductive strategy seems to be advantageous to aphids when we consider the number of descendants generated, since they produce larger offspring by reproducing asexually than sexually. But asexual reproduction in aphids is largely disadvantageous to host plants, once this mode of reproduction leads to a rapid accumulation of individuals, which can cause more intense and irreversible damage to host plants (Blackman & Eastop, 2021; Guerrero et al., 2013; Oliver et al., 2006; Sunnucks et al., 1996).

Aphid infestations of agricultural crops often require the implementation of pest control measures, and the use of insecticides is usually the most commonly taken (Foster et al., 2002; Ikbal & Pavela, 2019). However, the side-effects on non-target organisms and the environmental risks associated with the intense and excessive use of insecticides as a single strategy for pest control is often discussed (Costa, 2018; Debach & Rosen, 1991; Foster et al., 2002; Huffaker et al., 1976; Zhang et al., 2018).

Biological control of insects with natural enemies is an important strategy when multiple strategies for pest control are sought for joint implementation as an integrated pest management strategy to alleviate the selection pressure and the non-target effects associated with the sole use of organic insecticides (Baker, Green & Loker, 2020; Lucchi & Benelli, 2018; Zalucki, Adamson & Furlong, 2009). Parasitoids are among the most important natural enemies of insects, as they are capable of reducing populations of their pest hosts below the economic threshold level (Doutt, 1959; Eggleton & Gaston, 1990; Godfray, 1994; Kraaijeveld & Godfray, 2009; Russel, 1989; Thomson et al. 2010; van Lenteren, 2005). The parasitic way of life is found in different orders of insects (Diptera, Coleoptera, Lepidoptera, Trichoptera, Neuroptera and Strepsiptera), but is highly diversified in Hymenoptera, with 80% of the hymenopterans being parasitic wasps (Pennacchio & Strand, 2006).

The relationship established between host and parasitoid is the result of their coevolutionary history, leading to the selection of phenotypes of parasitoids that most successfully overcome the host defenses and exploit their host, and the selection of host phenotypes that carry successful behavioral, chemical, and/or physiological defensive mechanisms to escape parasitoid attack and/or the establishment of parasitization. The evolutionary process between host and parasitoid is explained by the red queen theory, which is based on the continuous development of adaptations to counteract the counter adaptations of the interacting group, resulting in co-evolutionary processes that select the best adapted individuals (Kraaijeveld et al., 2002, van Valen, 1977; Vienne et al., 2013).

The success of parasitoids depends on how elaborate and efficient the processes of host location, selection and exploitation used are to parasitize different hosts (Hafer & Vorburger, 2019; Vinson, 1976; Vinson & Iwantsch, 1980; Vinson, 1984; Godfray, 1994). Parasitoids developed different strategies of parasitization during their evolutionary history for the successful location of their hosts and the establishment and successful development of the offspring allocated to hosts (Vinson, 1976, Vinson 1984; Vinson, 1990). Koinobiont parasitic wasps mastered their weaponry to regulate several of the host's physiological processes in order to attend the physical and nutritional requirements of the immature parasitoid under development in the host (Harvey & Malcicka, 2016; Vinson & Iwantsch, 1980). The processes by which parasitic wasps regulate their hosts result in alterations of the host endocrine system, metabolism, and immune response to facilitate parasitoid successful colonization and development (Digilio et al. 1998; Falabella, 2018; Strand & Pech, 1995; Vinson & Iwantsch, 1980; Vinson, 1984; Vinson, 1990; Vinson et al. 2001).

The strategies host species developed to escape parasitization are based on parasitoid avoidance and immune defense. Host defensive strategies are based on three types of defense: 1) morphological (mimicry, camouflage), 2) behavioral (aggressive behavior), and 3) physiological traits (cellular and/or humoral immune responses) (Godfray, 1994; Greeney, Dyer & Smilanich, 2012; Gross, 1993; Kraaijeveld et al., 1998; Vilmos & Kurucz, 1998; Vorburger, 2014; Zhou, Meng & Li, 2017). Homochromy is a morphological defensive strategy many species of aphids employ to avoid attacks by natural enemies, as demonstrated by the more frequent attacks reddish-colored *Acyrthosiphon pisum* suffers from predatory ladybugs and parasitoids when compared to green-colored aphids (Losey et al. 1997). *Myzus persicae* is also able to avoid detection by natural enemies by adopting a body color similar to most of their host plant species (Gillespie et al., 2009).

There are several behavioral mechanisms associated with host defense against natural enemy attacks (Dixon, 2012; Firlej et al., 2010), and the most common behavioral defenses aphids display are the body rotation around the stylets while still inserted in the plant tissues, walking away, dropping from the plant, antennal jerking movements and/or confrontation (Dixon, 2012; Firlej et al., 2010; Gross, 1993; Le Ralec, et al, 2010; Stadler, Weisser & Houston, 1994). Such defensive mechanisms can be activated by visual perception, olfactory stimuli and/or perception of substrate vibrations, which indicate the presence of a parasitoid and very commonly leads to the release of alarm pheromones (Moayeri et

al., 2014). Release of alarm pheromones triggers a local response in other members of the colony that may respond by evading the area or by remaining in the area and display other defensive behaviors. The decision alarmed aphids take is based on the predicted costs associated with the location a new suitable host plant, which also represents risks for survival and reproduction (Firlej et al. 2010; Gross, 1993; Stadler, Weisser & Houston, 1994).

The third defensive mechanism consists in the activation and mounting of an immune response to isolate and eliminate the invader. Insects carry an innate immune system capable to provide humoral (melanization and antimicrobial peptides production) and cellular responses (phagocytosis, nodulation and encapsulation) (Gerardo et al. 2010). Encapsulation and melanization are the main mechanisms activated to eliminate multicellular invaders, such as the eggs of parasitoids. Encapsulation of parasitoid eggs by the host immune system results in parasitoid death by asphyxia and/or necrosis by the release of toxic and hydrolytic substances (Gross, 1993; Kraaijeveld et al. 1998; Vilmos & Kurucz, 1998; Vorburger, 2014). In some insects, including aphids, the immune system has been supplemented with toxins produced by associated-secondary symbionts, such as the ribosome inactivating proteins (RIP) produced by *Spiroplasma*, or the shiga-like toxin, the cytolethal distending toxin (CdtB) and the YD-repeat toxin produced by bacteriophages infecting the *Acyrthosiphon pisum* secondary endosymbiont, *Hamiltonella defensa*, which have been shown to improve insect immune response to entomophages and macrophages (Asplen et al. 2014; Ballinger & Perlman, 2019; Cayetano & Vorburger, 2015; McLean, 2019; Oliver et al. 2003; Rothacher et al. 2016; Rouil et al, 2020; Scarborough et al. 2005; Schimd et al. 2012). The mechanisms by which insect-associated bacteria enhance their host immune response against environmental stressors are still poorly understood. Nevertheless, the contribution of associated bacteria to host defense in the best studied system model was indicated to occur through 1) resources competition, in which host-associated microbial symbionts compete with natural enemies for limited nutritional resources (Paredes et al. 2016); 2) competition by interference, in which metabolites produced by the host-associated microbes can directly interfere with the survival and development of the natural enemy (Ballinger et al. 2017; Oliver et al. 2009); and 3) apparent competition, in which the symbiont can activate the host immune system (Kwong et al. 2017).

In aphids, several secondary symbionts were shown to interfere with the successful parasitization of aphids by parasitoids. The APSE bacteriophages infecting the bacterium *H. defensa* produce toxins of three distinct groups of proteins depending on the phage type (shiga-like toxin, CdtB, and YD-repeat toxin), resulting in increased parasitoid immature mortality (Martinez et al. 2016, Rouil et al, 2020). *Serratia symbiotica* is another secondary symbiont that interferes with production and emission of plant volatiles and affects host-plant attractance to aphid parasitoids (Frago et al., 2017; Oliver et al., 2003). The secondary symbionts *Regiella insecticola* (Vorburger et al., 2010) and *Candidatus* Fukatsuia symbiotica (Vorburger, 2018) were shown to interfere with the successful parasitization of aphids, but the mechanisms involved remain unknown. Aphids can still harbor several

other secondary symbionts (*Spiroplasma*, *Rickettsia*, *Arsenophonus* and *Wolbachia*), but their contribution to host defense have not yet been reported (Vorburger, 2018).

The defensive adaptations developed by insects to stressors is also associated with energy costs to build such response, which can carry associated fitness costs and affect fitness parameters, such as rate of growth, fecundity, fertility, and longevity (Boots & Begon, 1993; Kraaijeveld & Godfray, 1997; Martinez et al., 2018; Sager & Coley, 1995; Stadler, Weisser & Houston, 1994). Such defense mechanisms can be classified as constitutive or induced, and mechanisms that are constitutively available may require higher energy allocation (Schmid-Hempel, 2005). The existence of trade-offs between efficacious host defensive mechanisms to avoid parasitization and fitness traits due to associated costs can be decisive for the evolution and fixation of such defensive mechanisms in natural populations (Ebert, 2005).

The use of Classical Biological Control strategies in aphid biocontrol through the importation, multiplication and inoculation of parasitic wasps has been proven an efficient strategy by reestablishing the natural, ecological forces involved in the regulation of host – parasitoid populations, such as the use of parasitic wasps for aphid control in wheat fields in Brazil (Kenis et al, 2019; Stáry, Sampaio & Bueno, 2007; Sampaio et al, 2008). But the existing technologies for the mass production of insects and the establishment of biofabrics of biocontrol agents gave rise to Applied Biological Control strategies through the use of inundative releases of natural enemies (Oliveira et al. 2013). In such system, selected lines of biocontrol agents are successively mass produced and selected by responding to the selection pressures imposed by the rearing system they are exposed (Pinto & Stouthamer,1994), and used in ways to cause a shock effect on pest populations, representing a strong source of selection acting upon the target pest population. Since the stock population of natural enemies once selected for mass production and commercialization are maintained the same, we would expect that the continuous use of such "stable" selection pressure would lead to the selection of host phenotypes with increased capacity to avoid the successful attack by parasitoids and/or the establishment of parasitism, considering that the genetic variability of parasitoid species used in biological control programs is one of the factors that can determine the selection of defensive mechanisms against parasitization (Tomasetto et al., 2017; Tomasetto et al., 2018).

Palearctic and Nearctic species of aphids invasive to tropical and subtropical areas will not display the alternance from sexual and asexual reproduction modes naturally observed in the native regions due to the lack of the required environmental stimuli (Blackman & Eastop, 2021; Moran, 1992; Simon, Stockel & Tagu, 2010; Sorensen, 2009; Vorburger, Lancaster & Sunnucks, 2003). Thus, we would expect that populations of aphids invasive to tropical countries that are only reproducing asexually would carry very low genetic variability, once recombination does not occur in aphid parthenogenesis, and daughters are clones of their mothers (Blackman, 1979; Blackman & Eastop, 2021; Moran, 1992; Vorburger, Lancaster & Sunnucks, 2003). Therefore, we would expect to find no variation among sisters of clonal lines in response to parasitism, and that the expected differences among isolines

would allow us to better isolate and investigate the behavioral, physiological and molecular mechanisms behind aphid escape to parasitism.

Thus, in this dissertation we focused in evaluating the intra and interisoline variation in isolines of the invasive *Myzus persicae* in response to parasitization by *Diaeretiella rapae*, and select different phenotypes to investigate the existence of associated fitness costs and behavioral defensive mechanisms in response to parasitoid attack. Our data will contribute to the understanding of the mechanisms of parasitism avoidance aphids use, and support the development of investigations required to improve the successful use of parasitoids in applied biological control programs.

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2. VARIATION IN ISOLINES OF *MYZUS PERSICAE* TO SUCCESSFUL PARASITIZATION BY *DIAERETIELLA RAPAE* AND ASSESSMENT OF BIOLOGICAL TRAITS OF SELECTED LINES

ABSTRACT

Parasitoids are the main natural enemies of most insects, exerting strong selection pressure upon their hosts as successful parasitization implies in the elimination of the genetic pool of the parasitized host from the population genetic pool, once parasitized hosts die much more often before they reach their reproductive stage. The selection pressure imposed by parasitoids upon their living hosts leads to the selection of traits evolved to protect aphids from the successful development of parasitoids, and the exposure of parasitoids to host defense mechanisms leads to the selection of traits developed to counter-resist the host defense strategies. Thus, there are sources of chromosomal and extra-chromosomal variation, such as association with symbionts, that can influence the development of defense mechanisms by the host against natural enemies. The avoidance of parasitization may result in costs associated with the defensive mechanism. The coevolution in host-parasitoid interactions is difficult to observe in field populations since the visualization of resistant individuals depends on the host encounter and on the genotype of the natural enemy. Laboratory tests allow genotypes with greater and/or lesser capacities to avoid parasitoid attacks to be attacked at similar rates, under controlled abiotic and biotic conditions and similar patch structures, allowing host/parasitoid genotypes with selected responses to be studied. The present work evaluated the *Myzus persicae-Diaeretiella rapae* interaction under laboratory conditions to investigate the variation of isolines to parasitization and the existence of biological differences among selected clonal lines of *M. persicae* based on their association with secondary symbionts and successful parasitization by the parasitoid *D. rapae*. The observed success of parasitism in *M. persicae* by *D. rapae* varied between 43% and 76% in 14 tested isolines, of which six were selected (three in the first quartile = high parasitization; three in the fourth quartile = low parasitization) for the evaluation of biological parameters. The biological parameters evaluated showed significant differences between isolates of *M. persicae* with different responses to parasitism, and adaptive costs may be associated with the low parasitization observed in the selected isolines from the fourth quartile. The secondary symbiont *Rickettsia* does not provide additional defensive mechanisms to *M. persicae* when parasitized by *D. rapae*. Isolines of *M. persicae* carry variation within and among isolines in response to parasitization by *D. rapae* and in biological traits, but only one isoline clearly had costs associated with aphid capacity to avoid parasitization by *D. rapae*. Aphid infection by *Rickettsia* does not improve the defense response of *M. persicae* to parasitization by *D. rapae*, but it does increase female fecundity.

Keywords: Resilience to parasitism; Host-parasitoid interaction; Biological costs; Defensive symbiont.

2.1. Introduction

Ecological interactions are ubiquitous to all macro and microorganisms. Ecological interactions can be established between individuals of the same species (intraspecific interactions) or between individuals of different species (interspecific interactions). The main effects that an individual has on other individuals during their interactions can be positive for both, as in mutualism; negative for

both, as exemplified in competition, or positive for one and negative for the other individual, as in relationships of predation and parasitism (Leung & Poulin, 2008; Schoener, 1988; Travis et al. 2005).

In insect-insect relationships, parasitoids are the main entomophagous, and are exploited for conservative or applied biological control of different agricultural pests (Russell, 1989; Silveira et al. 2019). The term parasitoid was first used in 1913 by Reuter to define organisms with intermediate characteristics between predators and parasites (Eggleton & Gaston, 1990; van Lenteren, 2005; Vinson, 1976). Later, Doutt (1959) highlighted the major traits to differ parasitoids from parasites, arguing that parasitoids differ from parasites as they i) kill their hosts once they complete their immature development; ii) parasitize hosts belonging to their own taxonomic class; iii) are relatively smaller than their hosts; iv) mostly present a parasitic way of life only at their immature stage; v) complete their full development by exploiting a single host; and vi) regulate their host population in a way similar to predatory organisms (Vinson, 1976).

Unlike true parasites, parasitoids exert strong selection pressure on their hosts, because successful parasitization and parasitoid development leads to the elimination of the genetic pool the parasitized host represents from the population genetic pool, once parasitized hosts die much more often before they reach their reproductive stage (Godfray, 1994; Koltz et al., 2019; Kraaijeveld & Godfray, 2009; Moore et al., 2021). Thus, the selection pressure hosts face when exposed to parasitoids will lead to the selection of hosts that were unattractive to parasitoids and/or hosts that escaped parasitoid attack and survived parasitoid development. Insects that survive the attack and development of the parasitoid are observed mainly in interactions with koinobiont parasitoids than that presented with idiobionts, once idiobionts parasitoids induce the interruption of the host's development immediately after parasitism, or attack hosts in sessile stages such as eggs or pupae, while koinobionts, on the contrary, keep the host alive and in development, allowing host to feed and grow (Godfray, 1994; Harvey et al. 1994).

Koinobiosis is advantageous as parasitoids can attack hosts at earlier stages of development, favoring successful parasitization. Young hosts are known to have lower mechanical, physiological and behavioral defensive capacity, and the fact they are allowed to grow after parasitization permits the host to develop into a suitable nutritional resource even when they are parasitized at a sub-optimal nutritional stage (Jervis & Ferns, 2011; Kraaijeveld et al. 1998). But koinobiosis is also disadvantageous to parasitoids once parasitoids cannot access the future availability of nutritional resources to their hosts, and young hosts are more vulnerable to biotic and abiotic mortality factors. Nevertheless, parasitoids that keep their hosts alive can also count on the use of the host’s defensive strategies to protect the allocated parasitoid progeny to natural enemy attacks (Brodeur & Boivin, 2004; Jervis & Ferns, 2011; Kraaijeveld et al. 1998; Weinersmith, 2019). An example of this is the strategy used by most immature koinobionts parasitoids, in which the immature avoids carrying out processes that compromise the host's defense and mobility capacity, keeping the host functional until the end of its immature development (Brodeur & Boivin, 2004; Broudeur & McNeil, 1989; Weinersmith, 2019). Also, parasitoids can induce

behavioral changes in their hosts for their own benefit (Broudeur & McNeil, 1989; Brodeur & McNeil, 1990; Brodeur & Boivin, 2004; Muller, 1994; Weinersmith, 2019).

The selection pressure imposed by parasitoids upon their living hosts leads to the selection of traits evolved to protect aphids from the successful development of parasitoids, and the exposure of parasitoids to host defense mechanisms leads to the selection of traits developed to counter-resist the host defense strategies (Kraaijeveld & Godfray, 2009). The resulting coevolutionary processes during the evolutionary history of host – parasitoid interactions are reinforced by the theory of the red queen, in which each adaptation developed by one species faces a counter adaptation developed by the interacting species, in ways that the survival of both species depends on the continuous development of defense and attack strategies (arms-race) (Brockhurst et al. 2014; Kraaijeveld et al. 2002; van Valen, 1977; Vienne et al., 2013).

The coevolution in host-parasitoid interactions is frequency-dependent (Hamilton, 1990). Parasitoids are selected to avoid mechanisms of defense of common host genotypes, contributing to the selection of hosts with rare resistance genotypes (Carius et al. 2001). This process is difficult to observe in field populations since the visualization of resistant individuals depends on the host encounter and on the genotype of the natural enemy. Host genotypes with a lower capacity to circumvent the encounter with natural enemies are more frequently attacked. But genotypes of parasitoids will face similar selection pressure, resulting in a process of selection that will occur without the fixation of extreme genotypes of hosts or parasitoids (Barret, 1988; Carius et al. 2001). Laboratory tests allow genotypes with greater and/or lesser capacities to avoid parasitoid attacks to be attacked at similar rates, under controlled abiotic and biotic conditions and similar patch structures, allowing host/parasitoid genotypes with selected responses to be studied (Stiling, 1987).

The genetic variability that allows the manifestation of phenotypes with different capacities to respond to parasitoid attacks, as well as parasitoids with phenotypes with different levels of success in host parasitization, may be generated from many factors such as mutations, increased gene flow, genetic drift, inbreeding depression, and selection (Amos, 1998; Vellend & Geber, 2005). However, the main and quickest way to obtain genetic variability is through sexual reproduction, which allows the perpetuation of mutations in the population, as well as genetic recombination between chromosomes (Crow, 1994; Gerber & Kokko, 2016).

Aphids developing in regions of temperate climates display seasonal reproductive polyphenism, alternating from asexual to sexual reproduction in order to produce eggs that will diapause during the winter (Moran, 1992; Vorburger, Lancaster & Sunnucks, 2003; Shah et al., 2018). But under tropical conditions, aphid species such as *Myzus persicae* will reproduce exclusively by ameiotic (apomictic) parthenogenesis, in which chromosomal division occurs by mitosis. In apomixis reproduction the resultant progeny will be genetically identical to their mothers, and the sources of genetic variation to promote phenotypic expression are now limited to rare genomic events, such as mutations, chromosomal rearrangements, and mitotic recombination, and the interaction with

endosymbionts (Guerrero et al. 2013; Oliver et al. 2006; Sunnucks et al. 1996). Chromosomal rearrangements and interactions with symbionts have been shown to serve as sources of genetic variation leading to the manifestation of insecticide-resistant and host-plant adapted phenotypes (Blackman et al., 1995; Brown & Blackman, 1988; Kikuchi et al., 2012; Oliver et al., 2008; Russell & Moran, 2006). Thus, there are sources of chromosomal and extra-chromosomal variation that can influence the development of defense mechanisms by the host against natural enemies, which invariably carry associated adaptive costs (Gwynn et al. 2005; Rigby et al., 2002; Sunnucks et al., 1996; Vorburger et al. 2008).

Thus, we selected the association *M. persicae* - *Diaeretiella rapae* to investigate i) the variation of clonal lines to parasitization, ii) the association with secondary bacterial symbionts that could interfere in the host response to parasitization, and iii) and the existence of biological differences among selected clonal lines of *M. persicae* based on their association with secondary symbionts and successful parasitization by *D. rapae*.

2.2. Material and methods

2.2.1. Establishment and maintenance of aphid isolines

Aphid isolines were established from adult female aphids collected in cabbage plants (*Brassica oleracea* var. acephala) in experimental fields at ESALQ/USP (22º42'46.1"S; 47º37'37.8"W) and in home vegetables gardens in Piracicaba (22º42'38.041"S; 47º38"30.221") and Americana (22º44'20.216"S; 47º18'19.325"W), state of São Paulo, and from canola plants (*Brassica napus*) (28º13'51.809"S; 52º24'13.752"W), state of Rio Grande do Sul. Female aphids were isolated in plastic containers containing a leaf of Georgia cabbage (*Brassica oleracea* var. acephala) as a substrate for aphid feeding and reproduction. A total of 43 clonal lines were established. Insects were maintained under controlled laboratory conditions (20±2°C; 70±10% RH; photophase 14h), and cabbage leaves were weekly replaced.

2.2.2. Plants

Cabbage plants were obtained from seeds (TopSeed®) sown in seedling trays with 200 cells. Seedlings with 10 cm in height and presenting three to four true leaves were transferred to 550 mL containers with Tropstrato HT® substrate and cultivated in a greenhouse. Plants were sprayed biweekly with a leaf fertilizer (Home, Maxx Garden - composition: 150 g/L N, 80 g/L P, 80 g/L K, 400 mg/L B, 100 mg/L Co, 600 mg/L Cu, 500 mg/L Mn, 100 mg/L Mo, 1 g/L Zn, and 60 g/L chelating agent) to stimulate sprouting. Seedlings were also treated every other week with micro (412 mg/L $MgSO_4$, 360 mg/L KNO_3, 140 mg/L $NH_4H_2PO_4$, 4.2 μg/L Zn, 4.2 μg/L Cu, and 12.6 μg/L Fe) and macronutrient solutions (80 mg/L NH_4NO_3, and 900 mg/L CaN_2O_6).

2.2.3. Parasitoids

The parasitoid strain was obtained from mummified aphids collected in Piracicaba, state of São Paulo (22º42'46.1"S; 47º37'37.8"W). Aphid mummies were individualized in glass tubes (8x1cm) containing a droplet of honey to feed the emerging wasp. After emergence, couples were formed and allowed to mate for 24 h. After mating, females were placed in plastic cages (20x15x10 cm) containing cabbage leaves infested with aphids for parasitization for 48 h. Wasps were then removed, and nymphs were maintained in the cages for mummies formation. Mummies were collected and transferred to clean dishes lined with filter paper. A droplet of honey was applied at the internal side of the lid as a food source for the emerging adults. The emerging adults were allowed to mate, and mated females were once again used for aphid parasitization. After seven generations under laboratory conditions, the isoline with the best growth was chosen for conducting the experiments. The selected isoline was maintained under controlled conditions (20 ± 2ºC; 60 ± 10% RH; photophase 14h) and continuously reared in nymphs of 2nd and 3rd instars of *M. persicae* as earlier described.

2.2.4. Insects taxonomic and molecular characterization

Both aphids and parasitoids were subject to morphological identification and molecular characterization of a partial region of the mitochondrial *cytochrome oxidase I* gene (mtCOI). Parasitoid specimens were slide-mounted in Hoyer's medium and species identification was done based on taxonomic keys of Pereira (2005) and Kavallieratos and collaborators (2013). Aphids were identified after fundatrices laid several nymphs. Aphid adults were collected, slide mounted and identified following Blackman & Eastop (2000). Briefly, the specimens were heated to 40ºC in 95% ethanol for 2 min, transferred to 10% potassium hydroxide (KOH) and heated to 60ºC for 5 min. Afterwards, the specimens were immersed in distilled water, the abdomen was perforated with a micropin for cleaning the intracorporeal contents, washed twice in distilled water, transferred to acetic acid for 5 min, immersed in clover oil for 10 min, and slide mounted in Entellan (Merck). Slides were allowed to dry at 50ºC for 15 days, and specimens were subjected to identification under a compound microscope.

Adult aphids and parasitoids were individually subjected to total DNA extraction as described by Sunnucks & Hales (1996). Briefly, insects were removed from absolute ethanol, allowed to air dry and each specimen was individually macerated in 448 µL of digestion buffer (400 µL of TEN – 10 mM Tris pH 8, 2 mM EDTA pH8, and 400 mM $NaCl_2$; 40 µL 20% SDS; 160 µg of proteinase K) and incubated for 1 h at 55ºC. Then, 300 µL 5M $NaCl_2$ was added to the sample, mixed for 30 s and centrifuged at 14,000 *g* (30 min). The aqueous layer was collected, transferred to a new vial and 750 µL of isopropanol was added. This solution was incubated at -80ºC for 2 h. Subsequently, samples were centrifuged (14,000 *g* x 4 ºC x 30 min), the pellet obtained was washed in 1 mL cold ethanol, and

centrifuged again (14,000*g* x 4ºC x 5 min). The pellet was then washed twice in 1 mL 70% ethanol and centrifuged as before. The final pellet was dried at 45ºC for 15 min in a speed-vac concentrator and resuspended in 20 µL of autoclaved Milli-Q water.

The quality and integrity of the genomic DNA (gDNA) obtained was verified by gel electrophoresis using a 0.8% agarose gel slab containing 0.5 µg/mL of ethidium bromide in TAE buffer (40 mM Tris-Acetate and 1mM EDTA, pH 8.2), followed by visualization in a UV-transilluminator. gDNA samples were quantified in a spectrophotometer, and only samples with a A260/A280 ratio between 1.7 and 1.9 were stored at -20ºC for later use in PCR amplifications.

Aphid and parasitoid gDNA were used for the partial amplification of *COI* using sets of universal cited by Folmer et al. (1994) and specific primers developed in this study. The universal set of primers (LCO1490F 5'GGTCAACAAATCATAAAGATATTGG 3' and HCO2198R 5'TAAACTTCAGGGTGACCAAAAAATCA3') was used to amplify nearly 708 bp of the COI of the aphid and parasitoid, and an additional set of aphid-specific primers (MPG2066F 5'ACCTGTTCTAGCAGGTGCTA 3' and MPG2666R 5'ATGGAAATGGGCTACTACATAGT 3') were used for the amplification of a second amplicon that overlapped with the amplicon obtained with the universal primers, yield an almost complete COI sequence (1091 bp) (Table 1, Figure 1). The amplification reactions were conducted in a final volume of 25 µL, and contained 15-60 ng/ µL of gDNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.32 µM of each primer and 0.6 U of Taq DNA Polymerase ($^{@}$Gene Direx). Thermal cycling conditions used to universal set of primers in amplification of the cytochrome oxidase I (COI) gene of *M. persicae* and *D. rapae* were 94ºC for 2 min (1x); 94ºC for 1 min, 50ºC for 1 min, 72ºC for 1 min (35x); 72ºC for 5 min (1x) and thermal conditions used to aphid specific primer were 94ºC for 2 min (1x); 94ºC for 45 s, 48ºC for 45 s, 72ºC for 90 s (35x); 72ºC for 10 min (1x).

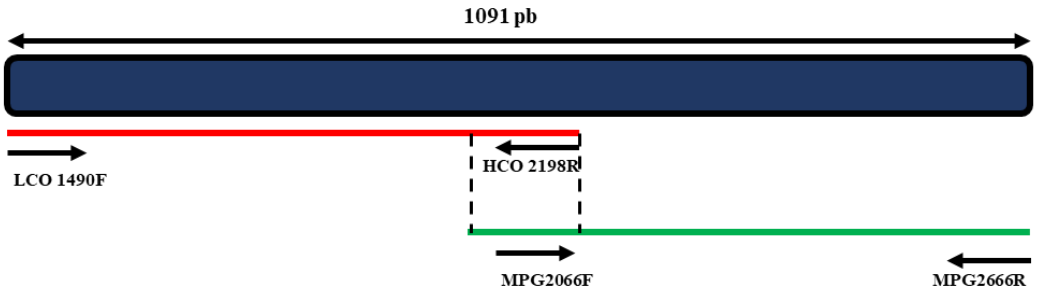

Figure 1: Representative illustration of strategy used for amplification of amplicons of the cytochrome oxidase I gene of *Diaeretiella rapae* and *Myzus persicae*.

Amplicons obtained from PCR reactions were visualized after gel electrophoresis on a 1.5% agarose gel slab, containing 0.5 µg/mL of ethidium bromide in TAE buffer (40 mM Tris-Acetate and 1mM EDTA, pH 8.2), using a UV transilluminator. Amplicons were then purified by adding 0.33U of shrimp alkaline phosphatase (SAP) and 3.3.U of exonuclease I (EXO) to 10 µL of the PCR reaction.

Samples were heated at 37ºC for 30 min followed by 15 min at 80ºC. Afterwards, samples were subjected to bidirectional Sanger sequencing using the original set of primers in a sequencing service provider.

Sequences obtained were analyzed and trimmed using Finch TV before heuristic searches against the nucleotide collection of the National Center for Biotechnology Information (NCBI) using BLASTn against the *nr* database. Sequences obtained for aphids and the parasitic wasp were individually aligned with the ClustalW tool (penalty of "gap" = 15, extension of "gap" = 6.66) with the closest matches obtained from our blast search, using MEGA version X (Kumar et al. 2018). Alignments were subjected to the determination of the most appropriate replacement models based on the lowest Bayesian information (BIC) and Akaike information (AIC) criteria as implemented in MEGA X. The evolutionary history was inferred using the Maximum Likelihood method based on the General Time Reversible model and gamma distribution pattern, and phylogenetic trees were built using the Maximum Likelihood method. The robustness of the branches was evaluated by the *bootstrap* method using 500 iterations.

2.2.5. Diagnostic-PCR detection of aphids secondary symbionts

Aphid gDNA was also used for the detection of associated symbionts using diagnostic PCR primarily based on the analysis of the *16S rRNA* gene of the most common symbiotic bacteria harbored by aphids (Table 1) (Haine, 2008; Moran et al., 2008; Oliver et al., 2010; Vorburger, 2018). PCR reactions contained 15-60 ng gDNA, 1x PCR Buffer, 2 mM $MgCl_2$, 0.2 mM each dNTP, 0.32 μM each primer, and 0.6 U Taq DNA Polymerase (Gene Direx) in a final volume of 25 μL. PCR conditions were optimized for each of the target symbionts (Table 1). Amplicons obtained from PCR reactions were visualized by gel electrophoresis as earlier described. The positive amplification of the primary aphid symbiont in the isolines under study was used to evaluate the quality of gDNA extraction.

Table 1. Specific primers and PCR conditions used in diagnostic PCRs for the identification of secondary symbionts associated with isolines of *Myzus persicae*.

Symbiont	Target gene	Primer	Sequence (5'-3')	Size (pb)	Thermal cycling conditions	Reference
Arsenophonus sp.	*16S rRNA*	16SA1	AGAGTTTGATCMTGGCTCAG	960	1x: 95ºC for 4 min; 40x: 95ºC for 30s, 55ºC for 30s, 72ºC for 30s; 1x: 72ºC for 6 min	Tsuchida et al. 2002
		Ars16SR	TTAGCTCCGGAGGCCACAGT			
Buchnera aphidicola	*16S rRNA*	Buch16S1F	GAGCTTGCTCTCTTTGTCGGCAA	430	1x: 95°C for 4 min; 40x: 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; 1x: 72ºC for 6 min	Tsuchida et al. 2002
		Buch16S1R	CTTCTGCGGGTAACGTCACGAA			
Candidatus Fukatsuia symbiotica	*16S rRNA*	PAXSF	AGTTTGATCATGGCTCGATTG	1200	1x: 94ºC for 5 min; 30x: 94ºC for 30s, 58ºC for 30s, 72ºC for 1 min; 1x: 72ºC for 5 min	Peccoud et al. 2014
		PAXSR	GCAACACTCTTTGCATTGCT			
Hamiltonella defensa	*16S rRNA*	T1279F	CGAGGGAAAGCGGAACTCAG	500	1x: 95ºC for 4 min; 30x: 94ºC for 1 min, 54ºC for 1 min, 72ºC for 1.5 min; 1x: 72ºC for 6 min	Oliver et al. 2005
		35R	CCTTCATCGCCTCTGACTGC			
Regiella insecticola	*16S rRNA*	U1279F	CGAACGTAAGCGAACCTCAT	700	1x: 94ºC for 4 min; 35x: 94ºC for 1 min, 58ºC for 1 min, 72ºC for 2 min; 1x: 72ºC for 6 min	Oliver et al. 2006
		35R	CCTTCATCGCCTCTGACTGC			
Rickettsia sp.	*16S rRNA*	16SA1	AGAGTTTGATCMTGGCTCAG	200	1x: 95ºC for 4 min; 40x: 95ºC for 30s, 55ºC for 30s, 72ºC for 30s; 1x: 72ºC for 6 min	Tsuchida et al. 2002
		Rick16SR	CATCCATCAGCGATAAATCTTTC			
Serratia symbiotica	*16S rRNA*	16SA1	AGAGTTTGATCMTGGCTCAG	1140	1x: 94ºC for 5 min; 30x: 94ºC for 30 s, 64ºC for 30 s, 72ºC for 1 min; 1x: 72ºC for 5 min	Niepoth et al. 2018
		PASS1140	TTTGAGTTCCCGACTTTATCG			
Spiroplasma sp.	*16S rRNA*	16SA1	AGAGTTTGATCMTGGCTCAG	510	1x: 95°C for 4 min; 40x: 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; 1x: 72ºC for 6 min	Tsuchida et al. 2002
		TKSSspR	TAGCCGTGGCTTTCTGGTAA			
Wolbachia sp.	*wsp*	wsp81F	TGGTCCAATAAGTGATGAAGAAAC	610	1x: 94ºC for 4 min; 35x: 94ºC for 30 s, 55ºC for 30 s, 72ºC for 30 s; 1x: 72ºC for 10 min	Braig et al. 1998
		wsp691R	AAAAATTAAACGCTACTCCA			

2.2.6. Assessing the parasitization of *Myzus persicae* isolines by *Diaeretiella rapae*

A total of 14 isolines of *M. persicae* were subjected to parasitization by *D. rapae*. Thirty 3rd instars of *M. persicae* of each isoline were placed onto 6 cm cabbage leaf disk laid onto a layer of 2% agar supplemented with 0.02% methyl parahydroxybenzoate (Nipagin®) in a Petri dish, and allowed to settle for one hour before exposition to parasitization by an experienced, host-fed, 24h-old mated *D. rapae* female. Each female wasp was used to parasitize up to seven nymphs randomly selected from aphid isolines. Nymphs were removed soon after the insertion of the parasitoid's ovipositor, inspected for the presence of the oviposition hole on the cuticle surface, and transferred to a new leaf disk. The leaf disks were replaced every 3-days to allow for aphid full development. The aphids were maintained on the leaf disk for a period of 7-10 days for mummification (=successful observed parasitization) or development of the aphid adult (=failure in parasitization or successful aphid defense). Mummified aphids were collected and transferred to glass tubes for adult parasitoid emergence.

Parasitism data were analyzed statistically by the Bernoullli generalized linear models, including the effects of lineage in the linear predictor. The significance of the lineage effect was assessed through the analysis of deviance. The selection of lineages that would be used in biological and behavioral assessments was based in the selection of three lines falling in the first (above the 75% percentile) and three lines in the fourth (below the 25% percentile) quartiles as representants of isolines with high and low parasitization rates, respectively.

2.2.7. Evaluation of biological parameters of isolines of *Myzus persicae*

The existence of adaptative costs associated with *M. persicae* differential response to parasitism by *D. rapae* was assessed on a selected set of clonal lines by the evaluation of biological parameters in the immature and adult stage of the host. Six isolines of *M. persicae* (three lines falling in the first and three lines in the fourth quartiles) were selected based on their response obtained in previous parasitism tests. Infection by the secondary symbiont *Rickettsia* was also considered.

The selected isolines were used for comparative analysis of fertility life tables. Biological data for the aphid immature development time (days) and survival (%) were obtained by setting up 16 replicates for each isoline. Each replicate was represented by a pool of 10 neonates infesting a potted cabbage seedling, totaling 160 nymphs/isoline.

Thirty newly emerged females were randomly selected from each isoline and individually placed on a cabbage plant. Females were daily observed for their survivorship and daily and total fecundity (number of nymphs produced) was recorded. The data obtained were used for the construction of fertility life tables to assess the reproductive success of each selected isoline by calculating and comparing the average interval between generations (T), finite growth rate (λ), intrinsic growth rate (Rm), net reproduction rate (Ro), and time for duplication (TD) (Krips et al. 1998; Rickelfs, 2003).

Plants and nymphs were maintained as before, and nymph mortality and adult development was checked daily.

Twenty adult females obtained from each isoline were also sampled and used for aphid size estimation by measuring the length of the left metathoracic tibia. Legs were removed from females, laid on top of a microscope slide and covered with a cover slide. Measurements were taken by using a stereomicroscope attached to a digital system for image capture and analysis (Motic Images Plus 2.0).

Aphid immature development time was analyzed by Cox proportional hazards models, using multiple comparisons ($p < 0.05$) as implemented in the RStudio statistical program (RStudio Team, 2020). Survival test based on the Kaplan-Meier method was used to analyze the probability of nymphs of *M. persicae* to reach adulthood ($p < 0.05$). The life table parameters were submitted to the resampling test by Jackknife, as referred by Maia & Luiz (2006), and subjected to statistical analysis after the use of Jackknife estimations of the means and variances for each parameter using the SAS based routine of Maia and collaborators (2000). The averages obtained were then subjected to post-hoc analysis using Tukey test ($p < 0.05$). Multivariate analyses were also used in the evaluation of life table data using multivariate linear models, including the effects of isoline as a linear predictor. The significance of the isoline effect was assessed using Pillai's trace test, and the heatmap was used to cluster isolines based on life table parameters using the Euclidean distance and Ward's method. Data on adult size were analyzed by ANOVA followed by Tukey test ($p < 0.05$) for average comparisons, using the RStudio statistical program.

2.3. Results

2.3.1. Insect identification and molecular characterization

Three aphid species were identified from the 43 isolines of aphids established: *Brevicoryne brassicae*, *Lipaphis pseudobrassicae* and *M. persicae* (Figure 2). They are common species in cruciferous plants and are differentiated by the presence or absence of wax covering the body, the relative size between the siphunculi and the aphid tail, and the presence of convergent or divergent frontal tubercles.

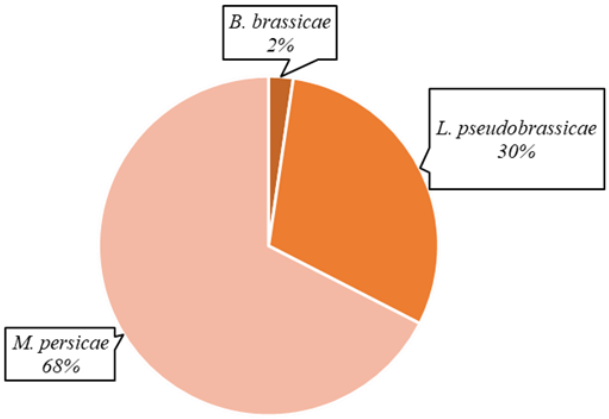

Figure 2. Percentage of isolines identified as *Brevicoryne brassicae*, *Lipaphis pseudobrassicae* or *Myzus. persicae*.

The analysis of COI sequences obtained for the isolines identified as *M. persicae* resulted in blast hits with 100% similarity with COI sequences belonging to this species. Phylogenetic analyzes produced a well-defined clade containing COI sequences of the isolines studied with others available in the *NCBI* database (Figure 3). The COI sequence analysis also demonstrated 100% similarity among the isolines sequenced, indicating that they can present the same maternal origin.

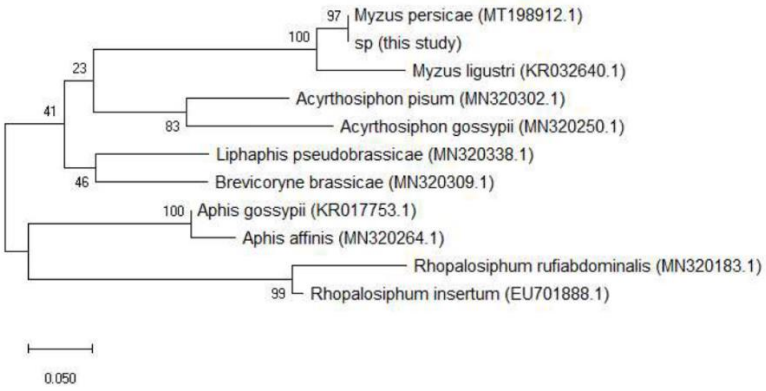

Figure 3. Phylogenetic tree of aphid species using partial sequences of the gene COI analyzed by the maximum-likelihood method. The scale bar indicates substitution by nucleotide position.

Taxonomic and phylogenetic analyzes based on COI information obtained for specimens of parasitoids collected identified the samples as *D. rapae*. The cluster of sequences obtained with sequences of *D. rapae* available in the *NCBI* database resulted in hits with 100% similarity with COI sequences belonging to this species. Phylogenetic analyzes resolved the COI sequence obtained in a clade containing only *D. rapae* (Figure 4).

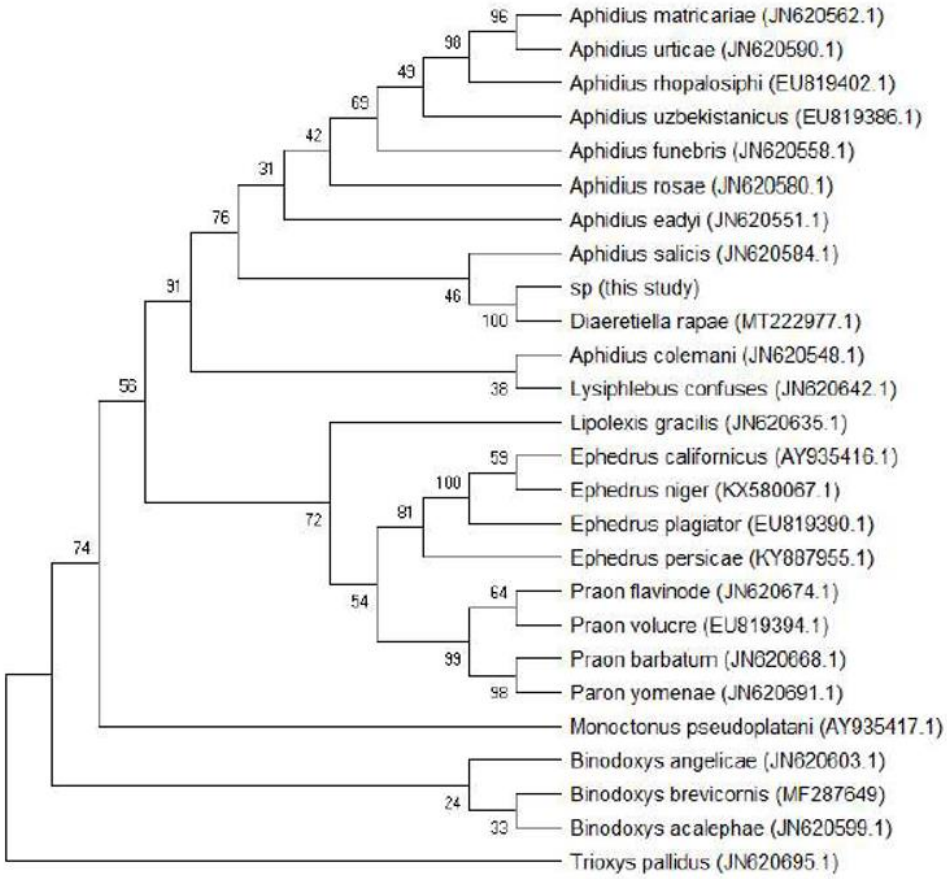

Figure 4. Phylogenetic tree of parasitoids species using sequence of gene COI analyzed by the maximum-likelihood method. The scale bar indicates substitution by nucleotide position.

2.3.2. Aphid symbionts detection

Only three out of the 14 isolines of *M. persicae* tested did not carry secondary symbiont associations. *Spiroplasma* and *Rickettsia* were the only secondary symbionts associated with singly or multiple infected isolines of *M. persicae* (Figure 5).

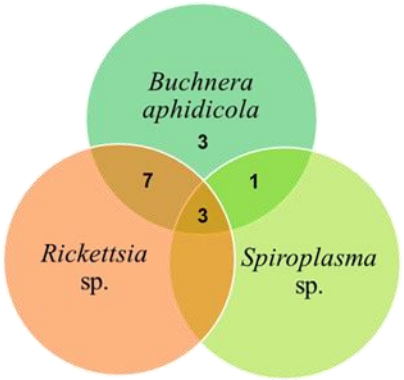

Figure 5. Clone lines of *Myzus persicae* used in parasitism test with infection by *Buchnera aphidicola*, *Rickettsia* and/or *Spiroplasma.*

2.3.3. Assessing parasitization of *Myzus persicae* isolines by *Diaeretiella rapae*

The observed success of parasitization of *M. persicae* isolines by *D. rapae* varied between 43% and 76%, with five isolines distributed above 75% percentile and five isolines below 25% percentile (Figure 6). In each one of these groups there were isolines infected or not with *Rickettsia*. Three isolines with average parasitization above 75% percentile (Iso2, Iso3, Iso4) (first quartile) and three isolates with average parasitization below 25% percentile (Iso10, Iso11, Iso14) (fourth quartile) were selected for further biological and behavioral analyses.

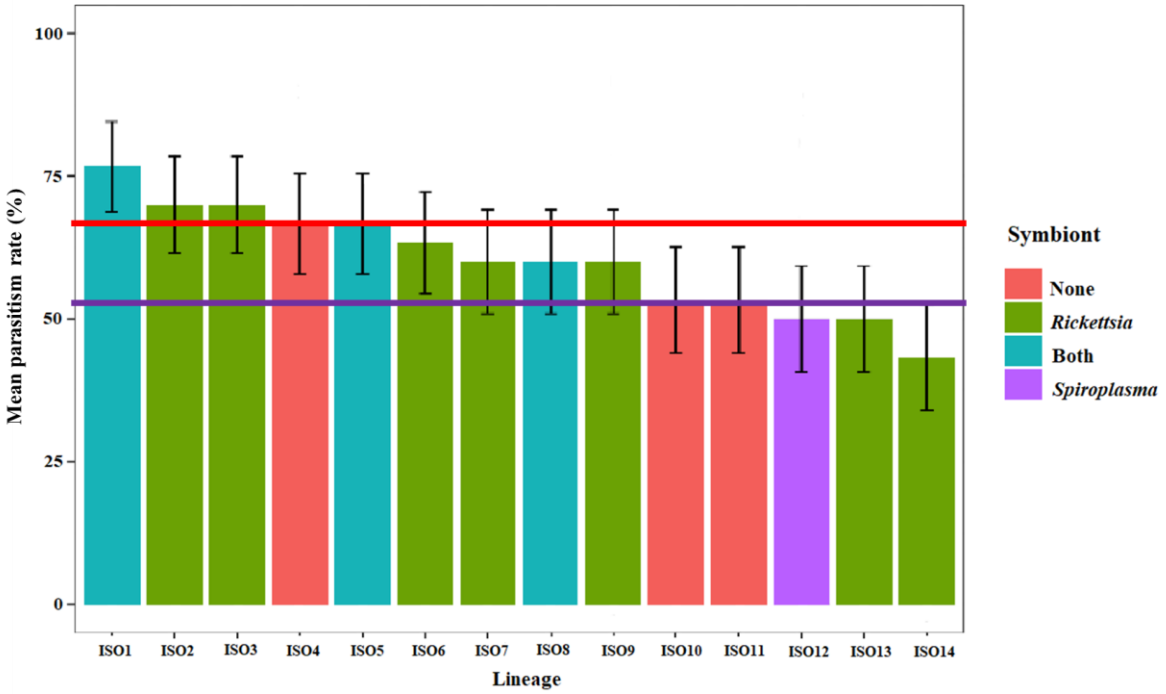

Figure 6. Successful parasitization of different isolines of *Myzus persicae* by *Diaeretiella rapae*. Red line = 75% percentile; purple line = 25% percentile.

2.3.4. Evaluation of biological parameters of isolines of *Myzus persicae*

2.3.4.1. Immature and adult biological parameters

The selected isolines of *M. persicae* differed in their immature survival (LR=15.94, *df*=5, *p*=0.007). The lowest probability to reach adulthood was observed for isolines ISO11 (probability to reach adulthood = 0.6711) and ISO14 (probability to reach adulthood = 0.7875), the only ones to differ from the isoline with the highest probability to reach adulthood ISO10 (probability to reach adulthood = 0.8625) (Figure 7A). We also detected differences in the time of development immatures required to turn into adults (χ^2=63.23, *df*=5, $p < 0.001$). Development of ISO4 (higher parasitization group - HPG) was similar to ISO3 (lower parasitization group – LPG), and were the longest of all isolines. The

development time of ISO10 (LPG) and ISO11 (LPG) was intermediate, and only differed from the time of development observed for ISO14 (LPG). ISO2 and ISO3 (both HPG) were among those with the shortest time of development and differed from ISO4 (HPG) and ISO14 (LPG) (Figure 7B).

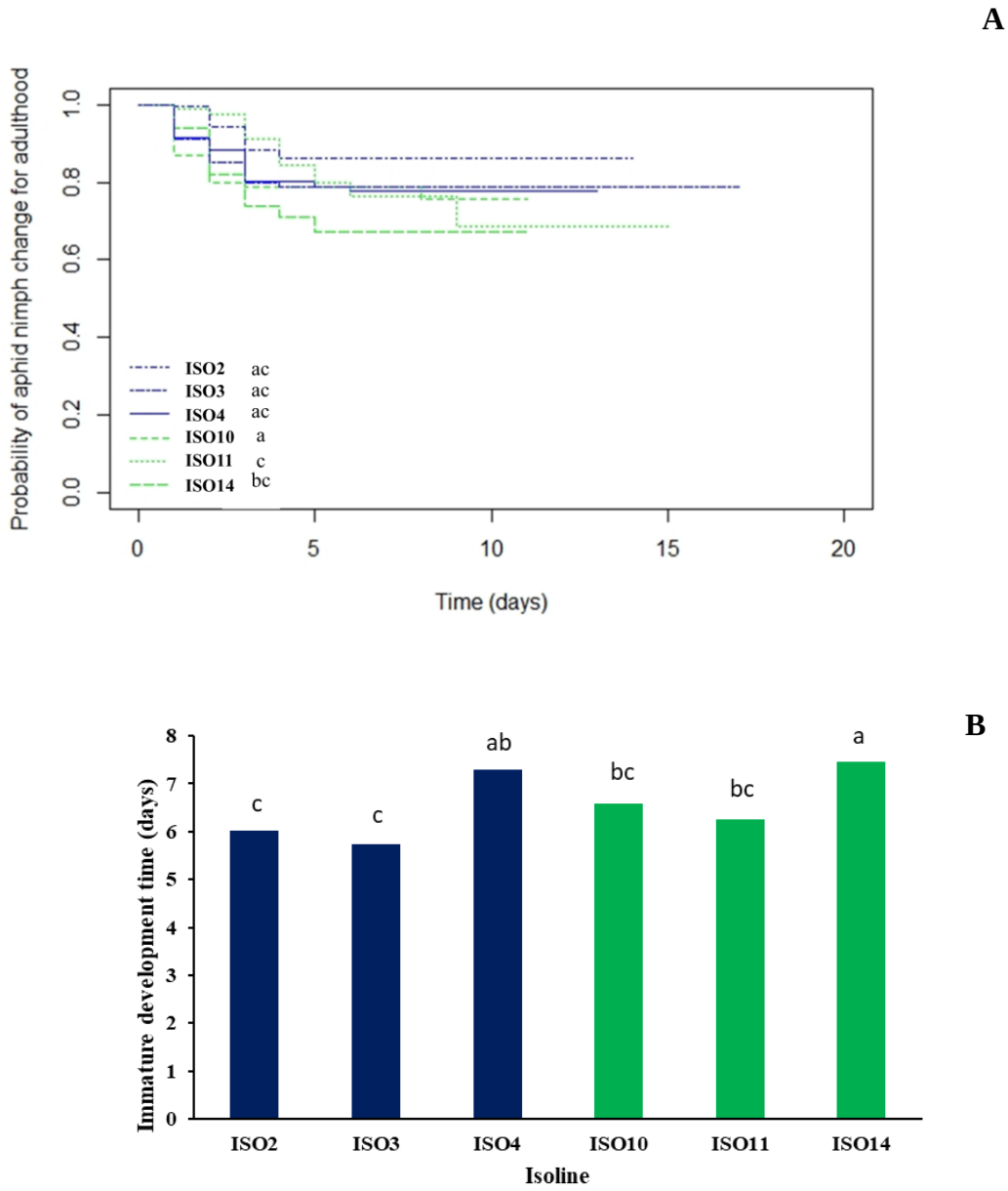

Figure 7. Immature development of *Myzus persicae* from selected isolines for high (ISO2, ISO3 and ISO4) and low (ISO10, ISO11 and ISO14) parasitization by *Diaeretiella rapae*. A) Probability of nymphs of *M. persicae* to reach adulthood. Treatments followed by different letters are statistically different (Cox proportional hazards models, $p < 0.05$). B) Immature development time. Treatments followed by different letters are statistically different (Kruskal-Wallis test. $p < 0.05$). Green bars= indicate the lineages of the group with lower parasitism. Blue bars= represent the isolines with higher parasitism.

The selected isolines also differed in their size ($F_{5, 112}$=3.62; p=0.0044), as estimated by the tibia size. Adult females of ISO2 being were larger than females of ISO4 and ISO14 (Table 2). No

differences in adult longevity were observed ($F_{5, 174}$=0.6167; p=0.6872), and differences in female fecundity were only detected between ISO10 and ISO11 ($F_{5, 174}$=2.53; p=0.027) (Table 2).

Table 2. Fecundity (nymphs/female), tibia size (µm) and longevity (days) of adult females from selected isolines of *Myzus persicae* for high (ISO2, ISO3 and ISO4) and low (ISO10, ISO11 and ISO14) parasitization by *Diaeretiella rapae.*

Isoline	Fecundity (nymphs/female)	Tibia size (µm)	Longevity (days)
ISO2	32.2±4.03^{ab}	1187.7±24.50^{a}	16.3±1.25^{a}
ISO3	30.0±3.76^{ab}	1095.6±42.20^{ab}	15.1±1.32^{a}
ISO4	25.6±2.98^{ab}	1036.7±16.20^{b}	15.8±0.99^{a}
ISO10	38.2±4.55^{a}	1123.1±29.30^{ab}	15.6±1.31^{a}
ISO11	20.1±2.37^{b}	1126.9±16.10^{ab}	13.8±1.05^{a}
ISO14	32.2±3.18^{ab}	1076.4±23.20^{b}	15.9±1.06^{a}

All selected isolines seemed to have a similar rhythm of reproduction, producing 80% of the total progeny between 13 and 15 days. But in two of the isolines that belonged to the first quartile of parasitization by *D. rapae*, such values of accumulated fecundity were obtained with much less than 80% of the reproducing females. In general, the pattern of reproduction in the isolines evaluated was similar among them (Figure 8).

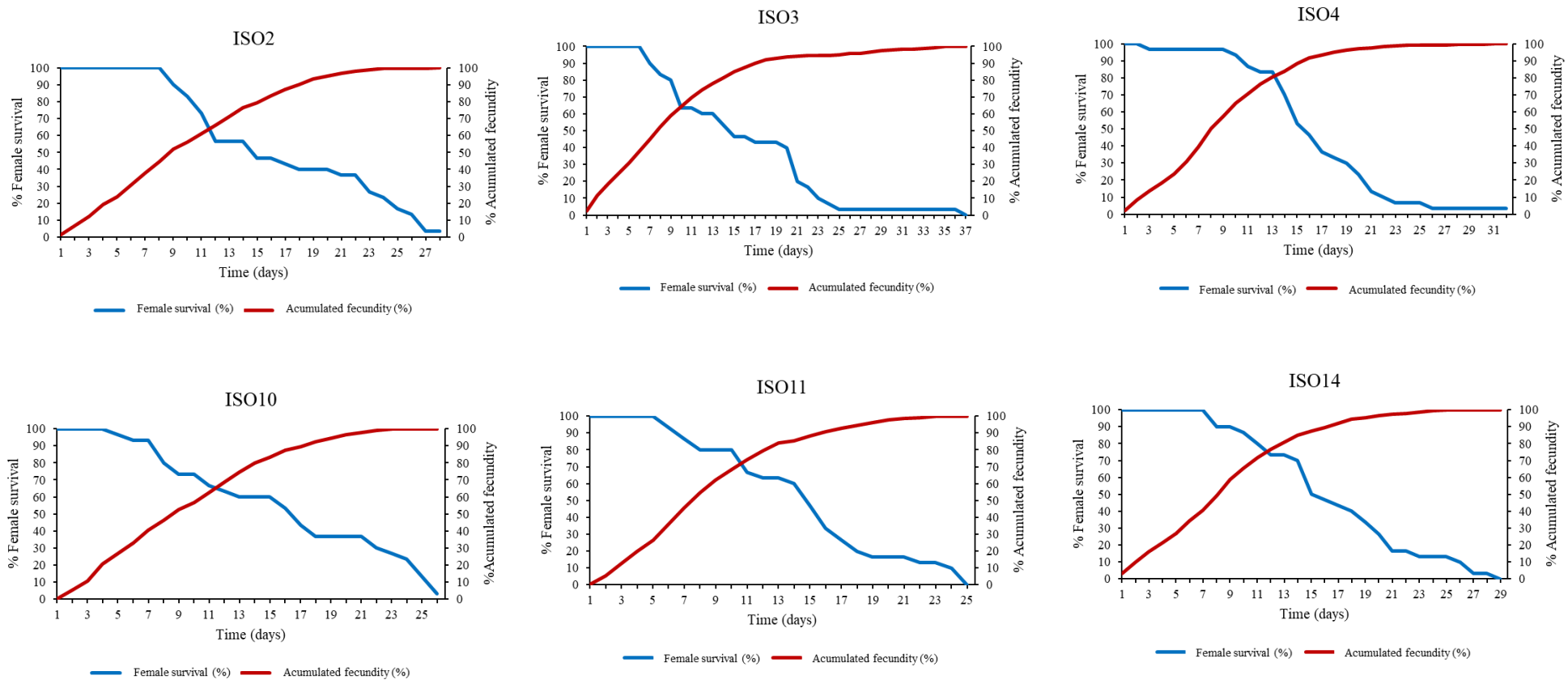

Figure 8. Daily female survival (%) and relative accumulated fecundity (%) of females of *Myzus persicae* from selected isolines for high (ISO2, ISO3 and ISO4) and low (ISO10, ISO11 and ISO14) parasitization by *Diaeretiella rapae*.

2.3.4.2. Fertility life table

Analysis of parameters of fertility life tables of isolines of *M. persicae* detected differences in the net reproduction rate (Ro) among the isolines studied ($F_{5, 174}$=5.425; p<0.001). Isoline ISO11 had the lowest *Ro* values, differing from ISO2 ($p=0.048$), ISO10 ($p<0.001$) and ISO14 ($p=0.038$), but not from ISO3 and ISO4, which had intermediate values (Table 3). The intrinsic growth rate (r_m) ($F_{5, 174}$=7.1119; p<0.001) and the finite growth rate (λ) ($F_{5, 174}$=7.0172; p<0.001) were higher for isolines ISO2, ISO3, ISO10 and ISO14 when compared to ISO11, and ISO3 also had higher values than ISO4 (Table 3). The interval between generations (*T*) observed for isoline ISO3 was shorter than those of ISO4 ($p= 0.037$), ISO10 ($p<0.001$) and ISO14 ($p= 0.025$), but similar to *T* obtained for ISO2 and ISO11. The time necessary for population to double in size for ISO11 line (3.46 d) was longer than for all other isolines, except ISO4 ($p<0.001$) (Table 3). The doubling time of ISO4 was also similar to isolines with intermediate *Dt* values, but different from ISO3 (Table 3).

Table 3. Life table parameters of selected isolines of *Myzus persicae* for high (ISO2, ISO3 and ISO4) and low (ISO10, ISO11 and ISO14) parasitization by *Diaeretiella rapae*.

Isoline	Fertility life table parameters				
	Net reproduction rate (Ro)	Intrinsic growth rate (r_m)	Finite increase ratio (λ)	Interval between generations (T)	Doubling time (*Dt*)
ISO2	25.0±13.99 ^{ab}	0.25±0.05 ^{ab}	1.28±0.06 ^{ab}	12.8±1.81 ^{ab}	2.8±0.50 ^{bc}
ISO3	23.4±13.67 ^{abc}	0.27±0.04 ^{a}	1.31±0.05 ^{a}	11.6±1.49 ^{b}	2.5±0.35 ^{c}
ISO4	18.9±9.05 ^{bc}	0.22±0.04 ^{bc}	1.25±0.05 ^{bc}	13.3±1.64 ^{a}	3.1±0.60 ^{ab}
ISO10	32.9±18.60 ^{a}	0.25±0.04 ^{ab}	1.28±0.05 ^{ab}	14.2±1.31 ^{a}	2.8±0.37 ^{bc}
ISO11	13.4±7.25 ^{c}	0.20±0.04 ^{c}	1.22±0.05 ^{c}	13.0±1.84 ^{ab}	3.5±0.66 ^{a}
ISO14	25.3±11.68 ^{ab}	0.24±0.03 ^{ab}	1.27±0.04 ^{ab}	13.3±1.87 ^{a}	2.9±0.40 ^{bc}

The correlation analysis and heatmap representation of the cluster analysis grouped isolines ISO4 and ISO11 in a well-defined cluster, and isolines ISO2 and ISO14 in internal subcluster of clusters with ISO3 and ISO10 (Figures 9 and 10). These clusters resulted from the sharing of closer values for *Ro*, *Rm* and *λ*, which were below the overall average for isolines ISO4 and ISO11 and above average for ISO10, ISO14, ISO2, and ISO3. The values of *Dt* and *T* for ISO10, ISO14, ISO2 and ISO3 were above average, while those of ISO 10 and ISO3 were below average (Figure 10).

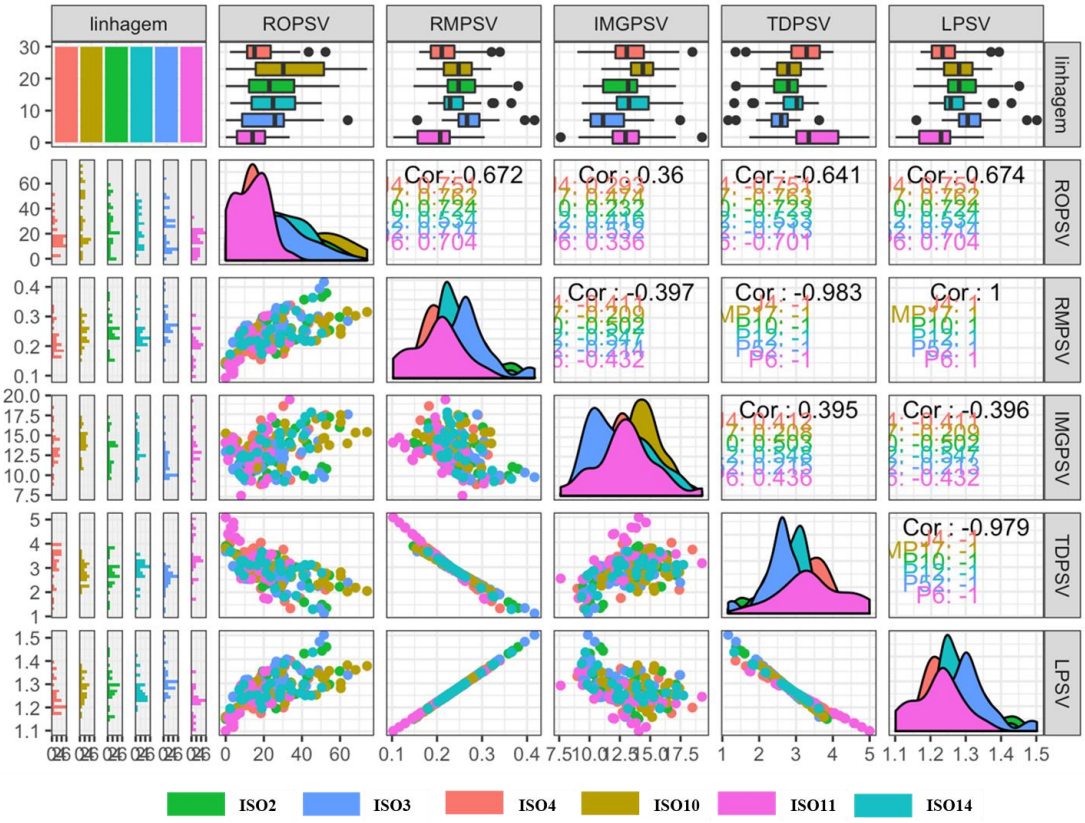

Figure 9.Pair correlation plot of fertility life table parameters of selected isolines of *Myzus persicae* with different responses to parasitization by *Diaeretiella rapae*.

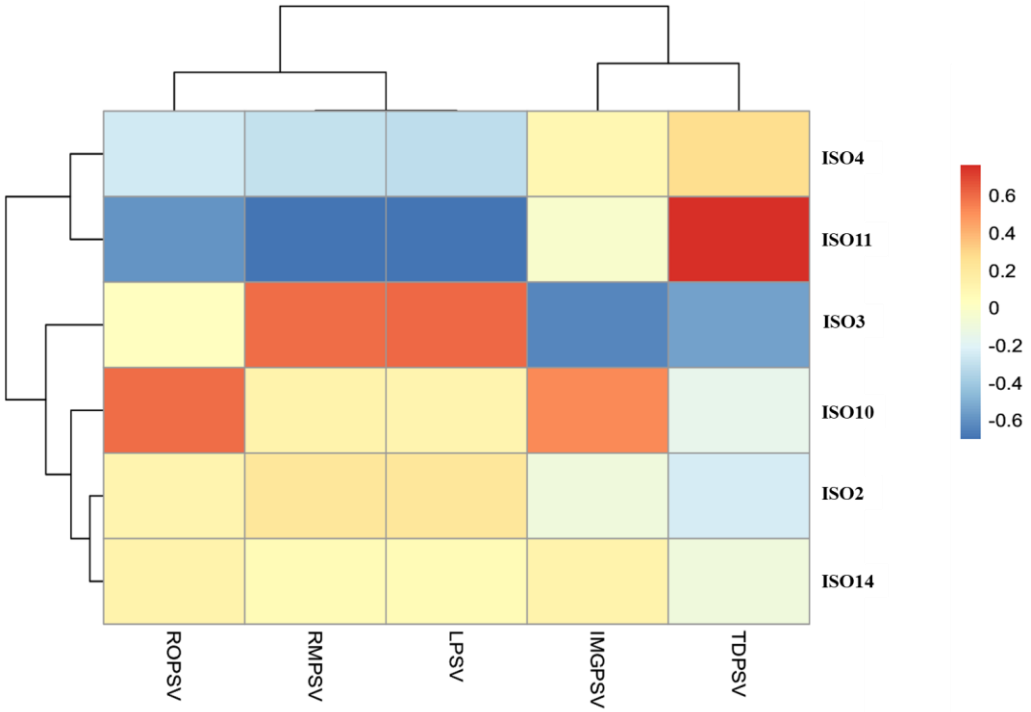

Figure 10. Cluster heatmap of reproductive life table data. The hierarchical clustering was generated using Euclidean distance and Ward's method. The color scale indicates the degree of correlation (white= low correlation; red= high correlation).

2.4. Discussion

Isolines of *M. persicae* presented high inter- and intra-isoline phenotypic variation in response to parasitization by *D. rapae*. *Myzus persicae* has also demonstrated phenotypic variation in color and size (Loxdale, 2008), ability to vector pathogens (Terradot et al. 1999), and in the susceptibility to biotic and abiotic factors (Losey et al. 1997; Loxdale, 2008). We did not expect to observe phenotypic variation in isolines of aphids reproducing through apomictic parthenogenesis, as the progeny originated by females are clonal in apomyxis (Sunnucks et al. 1996). However, genetic variation in clonal aphids was reported to occur due the high mutagenic capacity of DNA (Loxdale, 2010), chromosomal rearrangements, mitotic recombination and interactions with symbionts (Guerrero et al. 2013; Oliver et al. 2006; Sunnucks et al. 1996), and the genetic variability originated by such events would be able to produce phenotypic variation among individuals belonging to the same clonal lines. We also believe that the phenotypic variation within isolines could be associated with the differential expression of genes influenced by epigenetic mechanisms. Epigenetics can promote reversible and heritable traits to other generations, without changes in DNA nucleotide sequences (Rapp & Wendel, 2005).

The fact that the selected isolines carry the same mitochondrial marker, indicating they are all from the same maternal origin, the inexistence of association with host-defensive secondary symbionts, and the control of other factors that could interfere with the aphid response to parasitization (nutritional quality of the host plant, age of the aphid and the wasps, and the genetic variation of the parasitoid) (Hufbauer, 2001; Kumar, Kashyap & Soni, 2019; Wang, Chi & Liu, 2016), also indicates the isolines selected accumulated diversity sufficient to produce the phenotypic variability observed. Differences in density of the primary aphid symbiont *B. aphidicola* have been shown to interfere with the contribution of this symbiont and the energy-balance in aphis, resulting in the differential responses to parasitoids (Sakurai et al. 2005).

The manifestation of defense strategies regardless of their origin most often results in changes in biological parameters due to associated energy costs. Changes in energy allocation, with the use of higher amounts of energy to build a stronger immune system will result in a lower energy budget to be invested in life history traits, such as fertility or longevity (Gwynn et al. 2005). We did recorded differences in biological fitness traits among the isolines selected to represent aphids with high and low levels to respond to parasitoid attack and avoid the successful parasitization by *D. rapae*, as observed by Gwynn et al. (2005) and Vorburger et al. (2008), but contrary to von Burg et al. (2008). However, the association of fitness costs to the aphid response capacity to respond to *D. rapae* was not uniform in the two groups of aphids selected. Isoline ISO11, one of the isolines with parasitization levels by *D. rapae* in the 25% percentile, was the only isoline in this group to demonstrate reduced fitness in female fecundity and in several parameters of fertility life tables (Ro, rm, lambda) and a longer period to duplicate in number. In isoline ISO14, the delay in the development time was the only attribute affected. Lower growth rates are generally associated with lower metabolism to convert the ingest food into

energy (Calow, 1977; Sequeira & Mackauer, 1992), which is one of the determinants of the insect's nutritional quality (Sequeira & Mackauer, 1992).

Although ISO14 presents a delay in the development of the immature phase, no negative impacts were observed on the biological parameters evaluated for adults. The lack of negative effects in fecundity of isoline ISO14 could be associated with the infection of this isoline by *Rickettsia*. It is possible that *Rickettsia* could mitigate the costs associated with the higher capacity to avoid successful parasitization by *D. rapae* in this isoline, considering that this secondary symbiont has been shown to positively affect fecundity in other host – symbiont associations (Cass et al., 2015; Himler et al., 2011; Kliot et al., 2014; Kliot et al., 2019). Thus, the aphid fitness attributes observed in *Rickettsia*-infected isolines indicates *Rickettsia* does not benefit the response to parasization by *D. rapae,* but all *Rickettsia*-infected isolines had the highest values of fecundity. *Rickettsia*-infected aphids have been shown to have their association with the primary aphid symbiont affected and also to suffer fitness effects (Chen et al., 2000; Sakurai et al., 2005). The biological parameters we evaluated do not indicate *Rickettsia* could be interfering with *B. aphidicola* density in ways to affect the aphid nutritional quality to parasitoids (Chen et al., 2000), once *Buchnera* provides the aphid host with essential amino acids and vitamins in complementation of the nutrients obtained from host plants (Baumann et al. 1997; Rothacher et al., 2016; Silva et al., 1998; Viñuelas et al., 2007). Previous studies have shown that the association between insects and *Rickettsia* was beneficial to the host only in defense against entomopathogenic bacteria (Hendry et al., 2014).

We were not able to establish a relationship of aphid response to parasitization by *D. rapae* and the existence of associated fitness costs for isolines belonging to each one of the selected groups. The observation of fitness costs is dependent on the environment, and the adaptive cost varies depending on the environment conditions (Hunt et al., 2004), demonstrating that perhaps the conditions we used did not allow the expression of the costs associated with the differential capacity to respond to parasitoid attack. It is also possible that the selected isolines evolved different mechanisms to handle parasitoid attack, as aphid defensive mechanisms can rely in physiological, morphological or behavioral strategies (von Burg et al., 2008; Ferrari et al., 2001; Minchella, 1985; Sandrock et al., 2010; Vilcinkas, 2016), which may or not carry associated costs. Aphids defense against parasitization can also be provided by the differential metabolization capacity and utilization of secondary compounds obtained from their host plants to affect the successful establishment and development of natural enemies (Desneux, 2009; Gols & Harvey, 2008; Turlings & Benrey, 1998).

Our data demonstrated variation in aphid isolines to parasitization. Differences in the observed biological parameters and on the fertility life tables of the selected isolines suggests isolines may have invested in different mechanisms to handle parasitization by *D. rapae*, as isolines with successful parasitization rates in the 4th quartile did not follow a clear trend. *Rickettsia* infection did not influence the aphid response to parasitoid, but it seems to benefit aphid reproduction and to ameliorate costs associated with parasitism avoidance in at least one of the isolines tested (ISO14). Since the effects of

secondary symbionts to stress conditions may be dependent on the density of infection (Brown et al., 2012; Hopkins et al, 2017; Martinez et al., 2015;), further studies are required to proper investigate the contribution of *Rickettsia* to *M. persicae*. Further studies are required for the characterization of the defense mechanisms the selected isolines of *M. persicae* may have evolved to better respond and/or avoid the successful parasitization by *D. rapae*.

2.5. Conclusions

- There is variation in *Myzus persicae* isolines response to parasitization by *Diaeretiella rapae* within and among isolines*;*
- The selected aphid isolines have different biological traits, but only isoline Iso11 clearly had costs associated with aphid capacity to avoid parasitization by *D. rapae;*
- *Rickettsia* does not improve the defense response of *M. persicae* to parasitization by *D. rapae*, but it does increase female fecundity.

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3. BEHAVIORAL ANALYSIS OF THE INTERACTION OF *MYZUS PERSICAE* ISOLINES - *DIAERETIELLA RAPAE*

ABSTRACT

Aphids are sap-feeders of agricultural importance, damaging cultivated plants worldwide and causing millions of dollars of economic losses. Natural enemies are important regulators of aphid populations and are commonly used in applied biological control programs of aphids. Natural enemies impose high selection pressure to the evolution of defense mechanisms to avoid predation or parasitism, since predators and parasitoids influence the density and population growth of prey/host insects. Throughout the evolution of host-parasitoid interactions, morphological (camouflage), physiological (humoral and cellular immunity) and behavioral (kicking, wiggling, confrontation) processes were developed by hosts to avoid parasitization. Host defense against natural enemies can also be enhanced by associated symbiotic bacteria, which can inhibit parasitoid development. In the same way aphids developed defense mechanisms against their natural enemies, parasitoids have also developed strategies to circumvent the host defense tactics to successfully locate and exploit their hosts. Understanding the mechanisms of aphid defense and parasitoid strategies to attack is essential for the successful selection and utilization of parasitoids in biological control programs. In order to investigate the phenotypic diversity in behavioral responses during host-parasitoid interactions, this work evaluated isolines of the aphid *Myzus persicae* that differ in the successful rates of parasitization by *Diaeretiella rapae* in order to test the predictions that the differences in successful parasitization are regarded to direct alterations in host defensive behavior or host alterations that would interfere with the attractance and/or suitability of *M. persicae* to *D. rapae*. Investigations were realized by recording the parasitoid patch exploitation and the defensive behaviors of aphids in patches composed of three-host aphids under controlled conditions (25±2ºC; 70±10% RH). The behavior of parasitoids and aphids was evaluated according to parameters commonly analyzed in the literature and associated to defensive behaviors and patch exploitation. We detected significant differences in isolines with low and high parasitism when we evaluated the occurrence of host evaluation. *Rickettsia*-infection influenced the intensity and duration of host body wiggling behavior and the number and duration of host attack by *D. rapae* females. Thus, *Rickettsia*-infections affect the defensive behaviors of *M. persicae* aphids, but also interfere with the host selection process of the parasitoid *D. rapae.*

Keywords: Defensive behavior; Host-parasitoid interactions; Defensive symbiont; Patch exploitation.

3.1. Introduction

Aphids are sap-feeders of agricultural importance to cultivated plants worldwide (Blackman & Eastop, 2021) Dedryver et al., 2010; Ellis et al., 1996; Hartbauer, 2010; Katis et al., 2007). The characterization of these insects as pests of cultivated plants is associated with the intensity of the damage caused, which can be directly (malformation of plant tissues, nutritional deficiency) or indirectly associated with insect feeding (reduced photosynthetic capacity, pathogen transmission) (Blackman & Eastop, 2021; Dixon, 1981; Ellis et al., 1996; Hartbauer, 2010; Ortiz et al., 2005). The parthenogenic reproduction of aphids allows fast population growth in a short period of time, increasing the damage to plants and making it difficult to control (Hartbauer, 2010; Simon et al. 2010; Sunnucks & Hales, 1996).

Natural enemies are important regulators of aphid populations and are commonly used in applied biological control programs of aphids. Understanding the mechanisms of aphid defense and parasitoid strategies to attack potential host is essential for the successful selection and utilization of parasitoids in biological control programs (Hartbauer, 2010).

Natural enemies impose high selection pressure to the evolution of defense mechanisms to avoid predation or parasitism, since predators and parasitoids influence the density and population growth of prey/host insects by eliminating those that have been successfully attacked, allowing the selection of phenotypes that have developed morphological, physiological and/or behavioral strategies of defense against natural enemies (Braendle & Weisser, 2001; Ninkovic et al. 2013). Recent studies have shown that aphids have developed defensive mechanisms in response to the risk of attack by natural enemies (Boullis et al. 2017; Fan et al. 2018; Schuett et al. 2015).

The defensive strategies developed by aphids against natural enemies can have short- or long-term responses (Fan et al. 2018). Short-term responses include activities that can be performed momentarily by herbivorous insects, and aim to alarm the colony and prevent parasitoid attacks, such as the release of alarm pheromone to warn other colony members and alter the natural enemies foraging behavior (Nault, 2013); body wiggling and kicking the parasitoid with the hind legs (Dixon, 1958); interruption of feeding activities and dispersion from the location threatened by the natural enemy (Dill et al., 1990); detachment of the plant to avoid the attack or reduce the probability of development of the immature inside the host (Dill et al. 1990; Muratori et al. 2014); grouping individuals to reduce the attack through the dilution effect (Chacón & Heimpel, 2010); and select plants with no natural enemies at the time of colonization (Hopkins & Dixon, 1997). On the other hand, long-term responses consist of defense strategies to prevent future attacks by natural enemies, including, for example, ingestion of toxic allelochemicals to natural enemies (Verkerk et al., 1998), increase the development of winged individuals to improve the colony dispersion capacity (Sloggett & Weisser, 2002), and in some species, increase the production of soldiers for colony defense (Stern & Foster, 1996).

Short- and long-term responses can still be classified into morphological, physiological, or behavioral mechanisms (Gross, 1993; Schuett et al. 2011; Weisser et al. 1999). Morphological adaptations range from homochromy camouflage (Gross, 1993) to behavioral adaptations that include physical actions, such as kicking, wiggling and confrontation or escape from the attack of the natural enemy (Dill et al., 1990; Gross, 1993; Vorburger, 2014). Physiological adaptations evolved to avoid the establishment of parasitoids are targeted to eliminate invaders (bacteria, eggs, larvae) through the activation of cellular and/or humoral immune response pathways (Gross, 1993; Kraaijeveld et al., 1998; Vilmos & Kurucz, 1998; Vorburger, 2014).

Host defense against natural enemies can also be enhanced by associated symbiotic bacteria, which can inhibit parasitoid development by competing for limited resources available in the host (*eg*. lipids) (Paredes et al. 2016), producing toxins (Ballinger et al. 2017), and/or by the pre-activation of the host immune system (Kwong et al. 2017). Despite studies already carried out on defense mechanisms

against attack by natural enemies, physiological and behavioral strategies, as well as interaction with secondary symbionts, are still poorly understood (Desneux et al. 2009).

The host behavioral defense mechanisms can be activated upon contact with the natural enemy, detection of vibrations in the plant resulting from the foraging activity of the parasitoid/predator, and/or by the detection of alarm pheromone released by a colony-mate (Firlej et al., 2010; Losey et al., 1997). In the same way aphids developed defense mechanisms against their natural enemies, parasitoids have also developed throughout the coevolutionary history among these taxa, strategies to circumvent the host defense tactics to successfully locate and exploit their hosts (Vinson & Iwantsch, 1980). Successful parasitization depends on the efficacy of a sequence of processes associated with host location, selection, and parasitization, and the regulation of the host physiology to allow parasitoid immature development inside the host (Abram et al., 2019). The execution of the steps involving successful parasitization can impact host fitness, altering its behavior, development and reproduction, but it often results in the death of the host (Abram et al., 2019; Rehman & Powell, 2010).

Thus, we investigated isolines of the aphid *Myzus persicae* with different successful rates of parasitization by *Diaeretiella rapae* in order to test the predictions that differences in the successful parasitization are regarded to 1) direct alterations in host defensive behavior or 2) host alterations that would interfere with the attractance and/or suitability of *M. persicae* to *D. rapae*.

3.2. Material and methods

3.2.1. Insects

The insects used for behavioral analysis of the host-parasitoid behavioral interactions were obtained from previous experiments described in chapter two. These clonal lines were chosen based on successful rate of parasitization, differences in biology, associations with secondary symbionts, and *cytochrome oxidase I* (*COI*) molecular signature. They are divided in two groups. One group with the isolines that suffer high parasitization by *D. rapae* (Iso2, Iso3, and Iso4), and the second group with isolines with low parasitization rates (Iso10, Iso11, and Iso14).

3.2.2. Parasitism assays

The behavioral assays aimed to evaluate parasitoid and host behavioral patterns in uniform patches under controlled conditions (25 ± 1ºC, 70 ± 10% RH, 14 h photophase). The behavioral assays used three 3 d-old nymphs from each selected isolines and 24 h-old, naïve, mated females of *D. rapae* that were honey fed. Nymphs of *M. persicae* were placed onto 1.5 cm leaf disk of cabbage (patch) laid onto a slightly wet filter paper in a Petri dish (5 cm in diameter). Nymphs were allowed to settle for five hours before exposition to parasitization by *D. rapae*. One *D. rapae* female was released in the patch.

The behavior of aphids and parasitoids were recorded using a digital camera, and video analyses were carried out using BORIS v.7.9.19 (Friard & Gamba, 2016).

The parasitoid behavior was evaluated according to parameters commonly analyzed in the literature (Desneux et al, 2004; Outreman et al, 2001; Outreman et al, 2005; Tentelier et al, 2005; Zitelli, 2018; Wajnberg et al, 2006). The behavioral parameters observed for *D. rapae* evaluated for duration in seconds were: i) patch residence time; ii) time outside the patch; iii) parasitoid cleaning; iv) parasitoid resting; v) host searching (walking through the arena with antennal contact with the substrate); vi) host finding (when the parasitoid find the host); vii) host evaluation (parasitoid evaluate the host using the antennae) and viii) host attack (insertion of the ovipositor in the host). The number of occurrences of the following behaviors was also evaluated: i) cleaning behavior; ii) resting; iii) searching activity; iv) host finding; v) host evaluation and vi) host attack. The behavioral parameters analyzed for *M. persicae* were: i) body wiggling; ii) antennal whip – a defensive movement with the antennae; iii) kicking; iv) walking; v) abandonment of or return to the patch; vi) parasitoid confrontation. All behaviors observed for *M. persicae* were evaluated in terms of duration in seconds and the number of occurrences of each behavior. During the assays, the aphids were digitally numbered for further analysis of the behaviors presented by each tested specimen. Data collection started with the entry of the female parasitoid in the patch and lasted 10 min. A total of 20 replicates/treatment were used. Each patch with one female parasitoid was considered as one replicate.

Data were subjected to General Linear Models using multiple comparison ($p < 0.05$) using the average of each variable obtained for the three aphids attacked, with isolines nested within aphid parasitization (low *vs* high) and infection by *Rickettsia* (infected *vs* uninfected). The multiple comparisons of means were done using Tukey test ($p < 0.05$). All the statistical analysis were done in the RStudio statistical software (RStudio Team, 2020).

3.3. Results

3.3.1. Aphid and parasitoid behavior

The patch residence time of *D. rapae* was not affected by *Rickettsia*-infection or by the parasitization rate of the tested isolines by *D. rapae* (high and low parasitization rates) (Table 5). The time females of *D. rapae* spent on cleaning, resting, host drumming, host searching, host finding, and host evaluation were not affected by *Rickettsia* infection or the parasitization rate (Table 5). However, significant differences were found for the time *D. rapae* females remained outside the patch (F=3.71; df=3, 114; p=0.0137), the number of times parasitoid found hosts (F=4.61; df=3, 114; p=0.004) and displayed the cleaning behavior (F=3.12; df=3, 114; p=0.029) (Figure 11).

Table 4. Behavioral parameters of *Diaeretiella rapae* exploiting patches with selected isolines of *Myzus persicae* with high and low successful rates of parasitism, carrying ($Rick^+$) or not ($Rick^-$) *Rickettsia* infections.

Variables	Group	Average time (s)	Anova values
patch residence time	High	298.23±17.77	F=0.0060; df=1, 118; p=0.938^{ns}
	Low	299.99±14.98	
	$Rick^-$	307.48±15.70	F=0.5716; df=1, 117; p=0.451^{ns}
	$Rick^+$	290.74±17.06	
time outside the patch	High	269.75±19.74	F=0.0042; df=1, 118; p=0.948^{ns}
	Low	271.37±16.75	
	$Rick^-$	261.49±17.80	F=0.6239; df=1, 117; p=0.431^{ns}
	$Rick^+$	279.63±18.72	
cleaning	High	99.59±17.10	F=1.2706; df=1, 118; p=0.262^{ns}
	Low	74.98±13.23	
	$Rick^-$	84.95±14.59	F=0.0293; df=1, 117; p=0.864^{ns}
	$Rick^+$	89.63±16.11	
resting	High	5.52±2.38	F=1.0769; df=1, 118; p=0.302^{ns}
	Low	2.80±1.15	
	$Rick^-$	2.62±1.10	F=0.7729; df=1,117; p=0.381^{ns}
	$Rick^+$	5.70±2.40	
host searching	High	173.845±13.42	F=1.8457; df=1, 118; p=0.177^{ns}
	Low	197.13±11.10	
	$Rick^-$	196.72±12.50	F=0.8265; df=1, 117; p=0.365^{ns}
	$Rick^+$	174.26±12.14	
host finding	High	10.31±3.20	F=0.6977; df=1, 118; p=0.410^{ns}
	Low	7.50±1.18	
	$Rick^-$	11.42±3.30	F=3.54; df=1, 117; p=0.062^{ns}
	$Rick^+$	6.38±0.75	
host evaluation	High	15.25±5.50	F=0.3179; df=1, 117; p=0.574^{ns}
	Low	11.93±1.94	
	$Rick^-$	16.42±5.60	F=1.4847; df=1, 118; p=0.226^{ns}
	$Rick^+$	10.76±1.57	

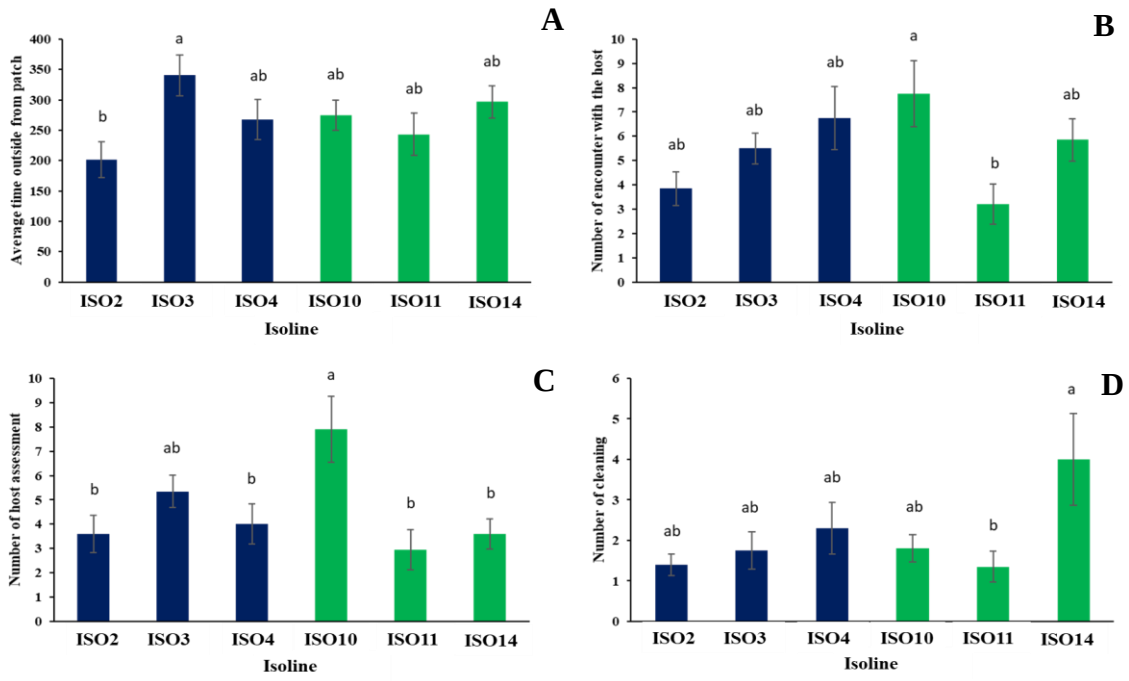

Figure 11. Behavioral parameters of *Diaeretiella rapae* exploiting patches with isolines of *Myzus persicae* selected from groups with high and low parasitism by *D. rapae*. A) Average time outside from patch; B) Number of encounters with the host; C) Number of host evaluations; D) Number of cleaning.

The number of attacks of female wasps in aphids of selected isolines was affected by their infection with *Rickettsia* (F=17.59; df=1, 117; p < 0.001) and the parasitization group (high vs low) (F=8.51, df=1, 118, p < 0.001). The average number of attacks by *D. rapae* was higher on *Rickettsia*-free *M. persicae* isolines (Figure 12A) and on isolines with low rates of parasitization by *D. rapae* (Figure 12A). The same pattern was observed for the time spent on this behavior (Figure 12B).

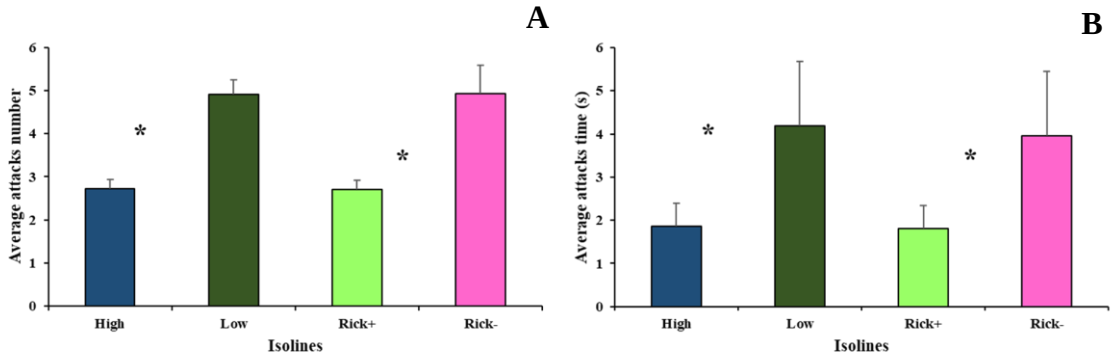

Figure 12. Average attacks of *Diaeretiella rapae* parasitoid in isolines of *Myzus persicae* from groups with high and lower parasitism and *Rickettsia*-free and *Rickettsia*-infected. A) Average attacks number by *D. rapae* in high (blue bars) vs low (dark green bars) and *Rickettsia*-infected (light green) vs *Rickettsia*-free (pink bars). * indicates statistical differences ($p < 0.05$).

The aphid body wiggling as a defensive behavior of aphids exposed to *D. rapae* females was affected by the aphid response to parasitism by *D. rapae* (low vs. high parasitization rate) and the association with *Rickettsia*. Aphids from isolines with a low parasitization rate by *D. rapae* responded

more intensively to parasitoid contact, but for shorter periods of time than aphids of isolines with high parasitization rates by *D. rapae* (Table 5). Aphids infected with *Rickettsia* displayed a higher number of body wiggling (agitation) but remained in that activity for a shorter period of time when compared to *Rickettsia*-free aphids (Table 5). Isoline ISO4 was the only isoline observed to display differences in behavior when compared to the remaining isolines. Once again, the only behavior that differed from all other isolines was the number and duration of body wiggling when parasitoids would contact the host (Figures 13A, B).

Table 5. Behavioral parameters of *Myzus persicae* evaluated for isolines classified into higher and lower parasitism groups and *Rickettsia*-free and *Rickettsia*-infected.

Variables	Group	Average	Anova values
# body movements (agitation)	High	13.26±2.34	*F=9.33; df=1, 118; p=0.0028*
	Low	24.20±3.04	
	$Rick^-$	16.09±2.79	*F=6.99; df=1, 117; p=0.0093*
	$Rick^+$	21.37±2.79	
Time invested in agitation (s)	High	180.15±29.39	*F=37.82; df=1, 118; p < 0.01*
	Low	58.09±7.13	
	$Rick^-$	183.36±29.26	*F=81.721; df=1, 117; p <0.01*
	$Rick^+$	54.88±6.70	
Time invested in antennal whipping (s)	High	0.13±0.06	*F=0.0592; df=1, 118; p=0.8082* ^{ns}
	Low	0.16±0.09	
	$Rick^-$	0.13±0.09	*F=0.1667; df=1, 117; p=0.6838* ^{ns}
	$Rick^+$	0.16±0.06	
Time invested in kicking (s)	High	1.74±0.73	*F=0.5904; df=1, 118; p=0.4439* ^{ns}
	Low	1.10±0.42	
	$Rick^-$	1.44±0.65	*F=0.1075; df=1, 117; p=0.7436* ^{ns}
	$Rick^+$	1.40±0.53	
Time invested in walking (s)	High	16.34±3.75	*F=1.2176; df=1, 118; p=0.2722* ^{ns}
	Low	11.36±2.55	
	$Rick^-$	15.70±3.70	*F=1.5811; df=1, 117; p=0.2112* ^{ns}
	$Rick^+$	12±2.65	
# of events abandoning / returning to the patch	High	0.094±0.031	*F=0.0745; df=1, 118; p=0.7854* ^{ns}
	Low	0.083±0.027	
	$Rick^-$	0.1±0.031	*F=0.4564; df=1, 117; p=0.50* ^{ns}
	$Rick^+$	0.078±0.027	
Time invested in confrontation (s)	High	0.011±0.0078	*F=0.00; df=1, 118; p=1.00* ^{ns}
	Low	0.011±0.0078	
	$Rick^-$	0.056±0.000	*F=1.1554; df=1, 117; p=0.2847* ^{ns}
	$Rick^+$	0.017±0.009	

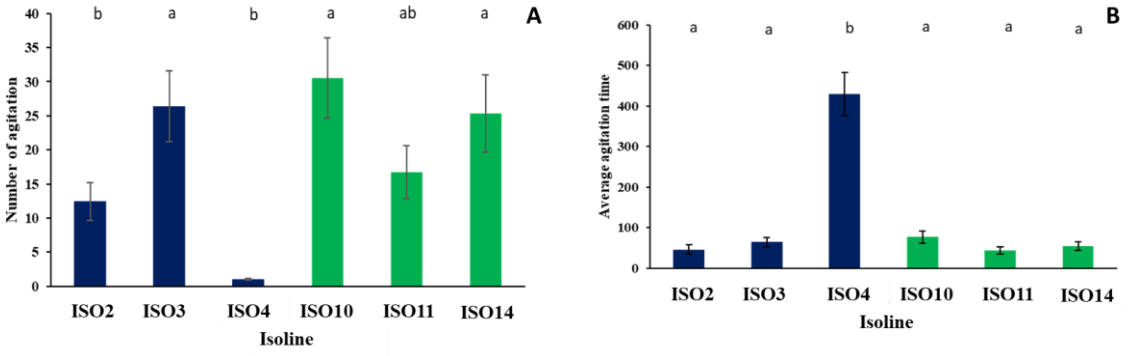

Figure 13. Intensity (n) (A) and duration (s) (B) of body wiggling as a defensive behavior of selected isolines of *Myzus persicae* for high (blue color - ISO2, ISO3 and ISO4) and low (green color - ISO10, ISO11 and ISO14) parasitism by *Diaeretiella rapae*.

3.4. Discussion

Parasitoids are important natural regulators of populations of insects, among which species of agricultural importance (Danneels et al., 2010). However, the success of parasitization depends on suitable biotic and abiotic conditions (Stary et al., 2007). In addition, physiological, behavioral and genetic factors of hosts and parasitoids can interfere with the outcome of the host – parasitoid interaction (Gross, 1993). The individual's behavior is often shaped by environmental conditions to which organisms must become adapted to (Shi et al., 2004) or to compensate other life history traits (Stamps, 2007).

The short life cycle of parasitoid species intensifies the importance of decisions to remain or leave a host patch, as well as the behavior performed in the patch in the presence of the host in order to optimize the foraging strategies adopted by the parasitoid (Wajnberg et al., 2015). We demonstrated through behavioral assays that several of the parasitoid behavioral parameters (average time out of the patch, number of encounters with the host, number of host evaluations, number of cleanings and average number and time of attacks) were affected in *D. rapae* when exploiting patches with selected isolines of *M. persicae* with high or low parasitization rates by *D. rapae*, and that were or not infected by the secondary symbiont *Rickettsia*.

Four main processes are necessary for parasitoids to be succeed in host parasitization: host habitat location, host location, host acceptance, and host suitability (Vinson, 1976). In our tests it was possible to analyze close-range behaviors involved in host location and in the evaluation and acceptance of the host for oviposition. The main factors that may be associated with these behaviors are the presence of visual and/or olfactory/gustatory cues that allow the location of the host (Du et al. 1998, Reyman & Powell, 2010) and the evaluation of the host quality (Mackauer, Michaud & Volkl, 1996).

Female wasps attacked more times aphids that were not infected by *Rickettsia* and aphids from selected isolines with a lower parasitization rate by *D. rapae*. The higher number of attacks that were required to parasitize aphids from the group with low parasitization rates by *D. rapae* might have been

required as aphids of this group had a much more intense defensive behavior through body wiggling. But *Rickettsia*-infected isolines also had a more intense body wiggling response to parasitoid contact, but suffered lower number of attacks, indicating *Rickettsia* infections may induce other changes to aphids that alter the preference of female wasps for aphids associated with this symbiont.

Studies demonstrate that secondary symbionts can promote changes in the host physiology, and that these changes are dependent on three main factors: species of the symbiont, genotype of the host or co-infection with other symbionts, as well as the density of the symbiont in the host (Liu & Guo, 2019). The physiological changes promoted in the host can be associated mainly with metabolic pathways, immune system, and gene expression, enabling adaptations that allow survival in the face of biotic and abiotic adversities, promoting, for example, the metabolism of xenobiotics (Liu & Guo, 2019) and defense against natural enemies (Frago et al., 2017; Martinez et al., 2016; Oliver et al., 2003; Vorburger, Genher & Rodriguez, 2010; Vorburger, 2018). In addition, symbionts can interfere with host and parasitoid interactions and affect the host selection and exploitation processes by parasitoids (Guo et al. 2017; Oliver et al., 2014; Vorburger, 2014, Vorburger, Ganesanandamoorthy & Kwiatkowski, 2013; Vorburger & Perlman, 2018), but few studies are dedicated to understand the role of secondary symbionts play on host behavior and on the interacting tritrophic level (Dion et al, 2011; Polin, Simon & Outreman, 2014; Ramírez- Cáceres et al., 2019; Sochard et al., 2020).

Although the physiological and behavioral alterations induced by secondary symbionts commonly associated with aphids have been described, little is effectively known on the induced changes that effectively leads to host protection against natural enemies (Guo et al., 2017; Vorburger, 2018). We observed a reduced number of parasitoid attacks in *Rickettsia*-infected aphids as compared to uninfected aphids, but with no behavioral display that would explain it. In fact, *Rickettsia*-infected aphids are more active in responding to parasitoid contact, as infected aphid aphids wiggle their bodies much more times. The stimulatory effect of *Rickettsia* has also been shown in ticks, with infected ticks displaying increased locomotory activity when compared to uninfected ticks (Kagemann & Clay, 2013). The lower number of attacks in refractory aphids suggests infected aphids are less attractive than uninfected aphids to *D. rapae*. *Rickettsia* symbiont can affect the host transcription (Martins et al. 2017) and lipid contents (Samanta et al., 2017; Ahyong et al., 2019), providing metabolic and physiological alterations in the host that could interfere with the process of host recognition (cuticular hydrocarbons) and selection (altered host quality, host energetics) (Fisher 1963; Liepert & Dettner 1996; Pan & Liu 2014; Sláma & Jedlicka 2012).

The behavioral parameters of female parasitoids that were observed to be affected when females were exploiting patches of selected isolines of aphids that have high or low parasitization by *D. rapae*, does not provide clear evidence that particular behavioral displays would explain the different success each group of aphids is parasitized by *D. rapae.* The recognition of behavioral displays that would explain different among isolines within each group of aphids (high and low parasitism) is even harder. But we did find evidence on the decisions *D. rapae* made in the exploitation of patches with aphids from

isoline Iso2, that suggests that parasitoids spent less time outside the patch and had lower frequency of host evaluation due to the fact aphids of Iso2 have larger body size (see Chapter 2). Body size is usually an indicator of host quality (Kouamé & Mackauer, 1991; Chau & Mackauer, 2001), and patches with high quality hosts are more attractive to parasitoids to search for hosts (Desneux et al. 2009; Du et al. 1998, Reyman & Powell, 2010).

In conclusion, the behavioral analysis of parasitoids and aphids demonstrated the major factors analyzed (symbiont infection and parasitization rate by *D. rapae*) interfered with some of the behavioral parameters of *D. rapae* and *M. persicae* evaluated. But we believe that only the increased intense of the host body wiggling can be a factor influencing the parasitization rates observed in for the group of isolines of *M. persicae* with low parasitism. Our data also demonstrates the need to search for additional sources of defensive mechanisms that could explain the parasitization observed in the two selected groups of isolines, particularly those involved with the immune response and metabolism of the aphid isolines.

3.5. Conclusions

- Infection of *Myzus persicae* by *Rickettsia* affects the aphid defensive behavior against *Diaeretiella rapae;*
- Infection of *M. persicae* by *Rickettsia* affects the patch time exploitation of *D. rapae;*

Rickettsia-infected aphids wiggle their bodies more intensively, but for a shorter period of time than *Rickettsia*-free aphids as a response to *D. rapae* attack;

- The more intense body wiggling of aphids can be the factor influencing the successful parasitization of aphids belonging to the group with low parasitism by *D. rapae.*

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