



GLEICE ANE DE SOUZA GONÇALVES

**REPRODUCTIVE ATTRIBUTES EVALUATION OF *Tuta absoluta* (MEYRICK, 1917) (LEPIDOPTERA: GELECHIIDAE),
WITH A FOCUS ON FEMALES' PREFERENCE AND MALES'
QUALITY**

**LAVRAS – MG
2024**

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PREFERENCE AND MALES' QUALITY**

Tese apresentada à Universidade Federal de Lavras,
como parte das exigências do Programa de Pós-
Graduação em Entomologia para a obtenção do
título de Doutor.

Prof. Dr. Khalid Haddi
Orientador

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LAVRAS – MG
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**AVALIAÇÃO DE ATRIBUTOS REPRODUTIVOS DE *Tuta absoluta* (MEYRICK,
1917) (LEPIDOPTERA: GELECHIIDAE), COM FOCO NA PREFERÊNCIA DE
FÊMEAS E NA QUALIDADE DOS MACHOS**

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como parte das exigências do Programa de Pós-
Graduação em Entomologia, área de concentração em
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À família Gonçalves, esta trajetória é, verdadeiramente, fruto do nosso vínculo e união.

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RESUMO GERAL

Tuta absoluta (Meyrick, 1917), uma das principais pragas das plantações de tomate, resulta em expressivos danos econômicos apesar das técnicas de controle empregadas, como o uso de armadilhas de feromônio. As persistentes perdas econômicas podem estar associadas ao comportamento reprodutivo dessa espécie. Diante disso, este estudo teve como objetivo compreender o comportamento reprodutivo da *T. absoluta*, explorando o efeito de características da qualidade dos machos, levando em conta a idade, peso e estado de acasalamento, nas escolhas das fêmeas e como essas escolhas e o múltiplo acasalamento impactam os principais parâmetros reprodutivos, incluindo oviposição, fertilidade e longevidade. Os resultados destacaram a preferência das fêmeas por machos de qualidade reprodutiva superior, especificamente jovens, virgens e mais pesados. Notavelmente, a qualidade do macho, especialmente em termos de peso, demonstrou correlação com o aumento da longevidade das fêmeas, embora não tenha influenciado significativamente a qualidade espermática. Surpreendentemente, observou-se uma maior quantidade de espermatozoides eupirenos em machos provenientes do ambiente natural. Além disso, o estudo revelou que múltiplos acasalamentos não exercem impacto significativo na oviposição, fertilidade ou longevidade das fêmeas de *T. absoluta*. A análise conjunta destes dados sugere que o efeito da qualidade dos machos, pode agir de forma mais sutil quando relacionada a escolhas das fêmeas e dos múltiplos acasalamentos sobre os parâmetros reprodutivos. Esses benefícios podem operar por meio de mecanismos indiretos e efeitos genéticos na aptidão da prole. A complexidade desses fenômenos ressalta a necessidade contínua de pesquisa para a melhor compreensão dos impactos a longo prazo na dinâmica populacional e evolução do comportamento reprodutivo da espécie.

Palavras-chave: escolha de parceiro; poliandria; qualidade espermática; Traça do tomateiro.

GENERAL ABSTRACT

Tuta absoluta (Meyrick, 1917), one of the main threats to tomato crops, results in significant economic damages despite employing the existing control techniques, such as pheromone traps. The persistent economic losses may be associated with the reproductive behavior of this species. Thus, this study aimed to comprehend the reproductive behavior of *T. absoluta*, exploring the influence of male quality characteristics, taking into account age, weight, and mating status, on female choices and how these choices and multiple mating impact key reproductive parameters, including oviposition, fertility, and longevity. The results highlight the clear preference of females for males with superior reproductive quality, specifically young, virgin, and heavier individuals. Remarkably, male quality, especially in terms of weight, demonstrated a correlation with increased female longevity, although it did not significantly influence sperm quality. Surprisingly, a higher quantity of eupyrene sperm was observed in males from natural environments. Additionally, the study revealed that multiple mating does not exert a significant impact on oviposition, fertility, or longevity in females of *T. absoluta*. The combined analysis of these studies suggests that the effect of male quality may operate more subtly concerning female choices and multiple mating on reproductive parameters. These benefits may operate through indirect mechanisms and genetic effects on offspring fitness. The complexity of these phenomena underscores the ongoing need for research to better understand the long-term impacts on the population dynamics and evolution of the reproductive behavior of the species.

Keywords: mate choice; polyandry; sperm quality; Tomato leafminer.

INDICADORES DE IMPACTO

Diante do desafio de alimentar uma população mundial que deve ultrapassar 9 bilhões de pessoas até 2050, a agricultura assume um papel central. A cultura do tomate destacando-se tanto pela sua importância nutricional quanto pelo seu valor econômico. A produção sustentável e rentável de alimentos como o tomate é essencial para garantir a segurança alimentar e a estabilidade econômica. No entanto, essa atividade enfrenta desafios significativos, como o controle da *Tuta absoluta* (Meyrick, 1917), uma das pragas mais devastadoras para as plantações de tomate. A presença dessa praga causa altos danos econômicos, afetando tanto pequenos agricultores quanto grandes produtores, apesar do uso de técnicas avançadas de controle, como as armadilhas de feromônio. Essas armadilhas são um exemplo de inovação tecnológica na agricultura, permitindo a captura seletiva dos insetos e reduzindo a necessidade de pesticidas químicos, promovendo práticas agrícolas mais sustentáveis e ecologicamente corretas. No entanto, as persistentes perdas econômicas associadas à *T. absoluta* podem ser atribuídas ao comportamento reprodutivo dessa espécie, que apresenta alta capacidade de adaptação e resistência, tornando o manejo da praga um desafio contínuo. Investir em pesquisa e desenvolvimento de novas estratégias de controle é essencial para mitigar esses impactos e garantir a viabilidade econômica da produção de tomate. Além dos benefícios econômicos e tecnológicos, a produção de tomate também possui significativos impactos sociais, gerando empregos e promovendo a inclusão social, especialmente em áreas rurais. Portanto, é necessário entender a ecologia e o comportamento reprodutivo do inseto alvo para entender os resultados obtidos no campo. Nesse contexto, compreender as características reprodutivas de *T. absoluta* e os aspectos comportamentais e fisiológicos desse inseto-praga é fundamental para prever o sucesso ou explicar possíveis falhas no controle, além de orientar diferentes estratégias sustentáveis de manejo da praga. Assim, estudos nesse sentido se tornam necessários para desenvolver práticas inovadoras que garantam a produção de alimentos de forma rentável, responsável e sustentável, assegurando a segurança alimentar global e o desenvolvimento socioeconômico.

IMPACT INDICATORS

Faced with the challenge of feeding a world population that is expected to exceed 9 billion people by 2050, agriculture assumes a central role. The tomato crop stands out both for its nutritional importance and its economic value. The sustainable and profitable production of foods such as tomatoes is essential to ensure food security and economic stability. However, this activity faces significant challenges, such as controlling *Tuta absoluta* (Meyrick, 1917), one of the most devastating pests for tomato plantations. The presence of this pest causes high economic damage, affecting both small farmers and large producers, despite the use of advanced control techniques, such as pheromone traps. These traps are an example of technological innovation in agriculture, allowing the selective capture of insects and reducing the need for chemical pesticides, promoting more sustainable and environmentally friendly agricultural practices. However, the persistent economic losses associated with *T. absoluta* can be attributed to the reproductive behavior of this species, which has a high capacity for adaptation and resistance, making pest management an ongoing challenge. Investing in research and development of new control strategies is essential to mitigate these impacts and ensure the economic viability of tomato production. In addition to the economic and technological benefits, tomato production also has significant social impacts, generating jobs and promoting social inclusion, especially in rural areas. Therefore, it is necessary to understand the ecology and reproductive behavior of the target insect to understand the results obtained in the field. In this context, understanding the reproductive characteristics of *T. absoluta* and the behavioral and physiological aspects of this insect pest is essential to predict success or explain possible control failures, in addition to guiding different sustainable pest management strategies. Therefore, studies in this sense are necessary to develop innovative practices that guarantee food production in a profitable, responsible and sustainable way, ensuring global food security and socioeconomic development.

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FIRST PART

GENERAL INTRODUCTION

Studying reproductive behavior is essential to understanding the evolutionary success of species, particularly in insects, where mate choice is a fundamental behavioral characteristic. Insects of both sexes exhibit traits or behaviors to maximize their reproductive success (ARNQVIST; NILSSON, 2000; TRIVERS, 2017). This choice is particularly significant in insect species displaying morphological and physiological variations within the same species, as these variations can influence mate preference, resulting in benefits for offspring (EBERHARD; CORDERO, 1995; JOVANOVIĆ et al., 2017).

In polyandrous species, where males mate with multiple partners throughout their reproductive lives, competition among males to attract females is intense (COLLET et al., 2012; PARKER; BIRKHEAD, 2013; MORIMOTO et al., 2019). While polyandry can offer reproductive benefits, such as genetic variability, it presents some associated costs, including increased risks of disease transmission and predation. These costs are crucial to consider when examining the complexity of reproductive strategies and their implications for species population dynamics (GOWATY; KIM; ANDERSON, 2012; PARKER; BIRKHEAD, 2013; MORIMOTO et al., 2019). In this context, males develop effective strategies to maximize reproductive success, while polyandrous females often prefer mating with males exhibiting traits suggesting higher reproductive quality. Multiple mating may be also associated with obtaining additional resources from males, such as nutrients and protection (BOGGS; GILBERT, 1979; WARD; LANDOLT, 1995; ARNQVIST; NILSSON, 2000).

In the context of *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae), a significant pest of tomato plants, a thorough understanding of its reproductive behavior is essential for the efficient control of this pest. The present work employed various approaches to provide a deeper insight into the reproductive behavior of *T. absoluta*. Regarding polyandry multiple mating does not significantly influence oviposition, fertility, and longevity compared to females who mated only once. This finding differs from what has been previously observed in some species (PERDIGUER et al., 1992; SETH et al., 2002; WALSH et al., 2011; LEE; ALBAJES; EIZAGUIRRE, 2014).

Some authors suggest that the impacts of polyandry may manifest more subtly, possibly through indirect genetic effects on offspring fitness. This may lead to individuals of superior quality avoiding genetic incompatibilities derived from inbreeding (KELLY; JENNIONS, 2011; PARKER; BIRKHEAD, 2013).

Similarly, the reproductive quality of an insect can also vary under the influence of biotic traits such as body size and weight, mating status, age, and the quantity and quality of spermatozoa (HONĚK, 1996; WEDELL; GAGE; PARKER, 2002; KELLY; JENNIONS, 2011; XU; WANG, 2020). Female insects exhibit a preference to mate with individuals of higher reproductive quality (PHELAN; BAKER, 1986; MARCOTTE; DELISLE; MCNEIL, 2005, 2007; COCCO; DELIPERI; DELRIO, 2012; MULLER et al., 2016). For instance, virgin and larger males provide a greater ejaculate, as well as a higher contribution to female fecundity and fertility through the transfer of nutritional reserves in the spermatophore (KELLY; JENNIONS, 2011; DE JESUS; REISKIND, 2016; HATALA; HARRINGTON; DEGNER, 2018). Our findings showed that *T. absoluta* females employ a partner selection mechanism, demonstrating a preference for young, virgin, and heavier males (GONÇALVES et al., 2024).

Other studies on *T. absoluta* indicate that body weight and virginity may be characteristics associated with the overall quality of males, as heavier and virgin males produce larger spermatophores, suggesting greater reproductive success (LÔBO, 2005; LEE; ALBAJES; EIZAGUIRRE, 2014; OLIVEIRA, 2020; SILVEIRA, 2020; HUAYHUA, 2022; GONÇALVES et al., 2024). Thus, males with these characteristics tend to be more successful in mating and sperm competition. On the other hand, it is suggested that females seek direct benefits from these males, such as increased fecundity, fertility, and improved offspring success (LINGREN; WARNER; HENNEBERRY, 1988; CALVO; MOLINA, 2005; MILONAS; ANDOW, 2010). Here, we observed that *T. absoluta* females mating with males of higher choice and considered of higher quality (virgin, young, and heavy) did not achieve greater oviposition or fertility although a direct positive influence of male body weight on female longevity was detected. Emphasizing the importance of understanding reproductive quality in the species' population dynamics, as reproductive behavior plays a role in developing effective pest management strategies, the information provided here points toward a careful selection of males for control programs as a strategy.

Sperm quality can also be related to reproductive quality in Lepidoptera (MARCOTTE; DELISLE; MCNEIL, 2005, 2007; KEHL et al., 2015). In this insect group, there is a dichotomous production of sperm, namely, the eupyrene sperm (nucleated) and the apyrene sperm (anucleated) (FRIEDLÄNDER, 1997; SWALLOW; WILKINSON, 2002; MANCINI et al., 2005; ALVES et al., 2006).

Both eupyrene and apyrene sperm originate from testicular cysts that divide synchronously and at the end of spermatogenesis are grouped in bundles that dissociate

immediately after mating completion (KOUDELOVÁ; COOK; KOUDELOVA, 2001; SETH et al., 2002). Although both types are transferred to females in the ejaculate, only the eupyrene sperm fertilize eggs (FRIEDLÄNDER, 1997; FRANÇA; BÁO, 2000; SWALLOW; WILKINSON, 2002; TILL-BOTTRAUD et al., 2005).

Reduction in female fertility can also be related to sperm quality (KEHL et al., 2015). Both sperm types (eupyrene and apyrene) can reach the female spermatheca (SWALLOW; WILKINSON, 2002) but the sperm ratio vary and can impact ejaculate characteristics throughout the male's life, depending on the mating context, as sperm production is a process that demands significant resources (OLSSON; MADSEN; SHINE, 1997; WEDELL; GAGE; PARKER, 2002). Previous studies indicated that apyrene cells are involved in sperm competition (MANCINI et al., 2005; MARCOTTE; DELISLE; MCNEIL, 2005, 2007). Eupyrene sperm are larger, thicker, and have a higher mitochondrial content than apyrene sperm; however,. apyrene sperm can account for up to 90% of the total of the two types (FRIEDLÄNDER, 1997; SWALLOW; WILKINSON, 2002). This variation, in turn, impacts female fertility (KEHL et al., 2015)

The tomato leafminer, *T. absoluta*, is a microlepidopteran widely recognized as one of the main pests affecting tomato plantations (GARCIA; ESPUL, 1982; GUEDES; PIKANÇO, 2012). The larvae of this species have an endophytic habit, leaving them protected in the leaf mesophyll or inside the fruits, reducing the efficiency of chemical products used in their control (COCCO; DELIPERI; DELRIO, 2012). To avoid or reduce the indiscriminate use of insecticides, control strategies based on sexual pheromone traps such as attraction, confusion, and mass trapping techniques are increasingly used in agriculture for pest control (LÔBO, 2005; PRASAD; PRABHAKAR, 2012). In experiments conducted with synthetic sexual pheromones for the behavioral control of this species, it was observed that despite the high suppression rate of males in the field, tomato production losses remained above the economic damage threshold (LÔBO, 2005; COCCO; DELIPERI; DELRIO, 2012; CAPARROS MEGIDO; HAUBRUGE; VERHEGGEN, 2013). The likely failures of the sexual pheromone trap system may be related to the polyandrous sexual behavior of the species, migration, and/or female competition with traps when in high population density (LÔBO, 2005; DAI et al., 2019)

A previous study with pheromone traps showed that the majority of collected *T. absoluta* males were mated (HUAYHUA, 2022). This may be related to male quality, with females preferring males considered of higher quality, including virgins. Thus, we propose that

males classified as lower quality may be more rejected by females, hence being more attracted to traps with synthetic sexual pheromones.

A comprehensive understanding of the reproductive behavior of the tomato leafminer is crucial for the development of efficient integrated management strategies. This involves everything from the discrete choice of partners by females to the impact of male body weight on female longevity, as well as the size of bundles of eupyrene sperm. It is also noteworthy that there is a higher proportion of these sperm in individuals from the field. This knowledge can be applied in creating more precise and adaptable strategies. The careful selection of males based on specific characteristics can be integrated into breeding and selective release programs, complementing existing practices, such as the probable release of sterile males. This approach aims to strengthen existing control strategies, making programs more efficient, sustainable, and tailored to the peculiarities of the reproductive behavior of this pest.

Thus, it is necessary to understand the ecology and reproductive behavior of the target insect to appropriately interpret the results obtained in the field. In this context, understanding the reproductive characteristics of *T. absoluta* males and the behavioral and physiological aspects of pest insects is necessary for predicting success or explaining possible control failures, as well as guiding different female receptivity strategies. One possibility would be to use research results to select sterile males with physical and behavioral characteristics similar to wild males.

FINAL CONSIDERATIONS

The diverse findings throughout this study provided a deeper understanding of the reproductive behavior of *T. absoluta*. In the first chapter, our findings confirm the hypothesis that *T. absoluta* females employ a mate selection mechanism. There was a clear preference for males with higher reproductive quality, especially those young, virgin, and heavier. This preference was primarily based on the males' body weight, directly influencing the females' longevity.

In the second article, the investigation into the effects of male body size on sperm quality revealed intriguing results. Contrary to expectations, no significant association was observed between male weight and the main quality analyses. However, heavier males produced larger bundles of eupyrene sperm, indicating a potential positive correlation between sperm size and sperm quality. This correlation emphasizes the importance of sperm characteristics in reproductive success. Additionally, the comparison between individuals from natural habitats and controlled environments showed a higher proportion of eupyrene sperm in the former, highlighting the environmental influence on *T. absoluta* sperm quality.

The third article, exploring the effects of multiple matings in *T. absoluta*, provided contrasting information. Unlike some studies, no significant differences were identified in the main reproductive characteristics between females who mated once and those subjected to multiple matings. This finding suggests that the impacts of polyandry may manifest more subtly, possibly through indirect genetic effects on offspring fitness, emphasizing the importance of standardization in experiments and isolating all factors that may influence study results. This integrated approach allowed for a deeper understanding of the reproductive biology of *T. absoluta*, highlighting the complexity of this behavior and underscoring the need for additional studies to be applied to more comprehensive and adaptive management strategies.

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SECOND PART: ARTICLES

ARTICLE I**Age, weight, and mating status of the male influence female choice and reproductive success in the tomato leafminer *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae)**

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Abstract

Mate choice is a fundamental behavior of polyandrous insects, in which individuals prefer mates that exhibit characteristic traits of higher reproductive quality. *Tuta absoluta* (Meyrick 1917) is the main pest in tomato cultivation and is difficult to control. Pheromones-based strategies; including monitoring, mass trapping and mating disruption; are recommended within integrated pest management of this pest. However, significant losses in tomato production are still reported despite the effective capture of males suggesting a potential failure of the used pheromones-based technologies in the field. As this situation may be related to the sexual behavior of this species, this study aimed to investigate how male's characteristics, such as body weight, mating status, and age, influence the reproductive behavior of *T. absoluta* females and thus understanding the reported efficacy losses. First, we conducted a set of tests in which females were allowed to choose between males of different weights, mating status, and age. The effects of mating with males presenting the most contrasting characteristics on the females, reproductive outputs and longevity were assessed. Our results show that females of *T. absoluta* prefer males with higher reproductive quality (young, virgin and heavy) and that the male's body weight had a direct influence on the increased longevity and reproductive performance of females. Understanding the reproductive success of these insects can help develop more effective control technologies to better reduce losses in tomato production caused by this pest.

Keywords: Fertility; Fecundity; Reproduction; Integrated Pest Management; Male Quality

Introduction

Mate choice is a critical behavioral in insects, in which females of both sexes display attractive traits or behaviors to maximize their reproductive success (Trivers et al. 1972; Arnqvist & Nilsson 2000). Both insects' sexes may exhibit morphological and physiological differences within the same species that could influence mate choice and may confer benefits to offspring (Eberhard & Cordero 1995; Jovanovic et al. 2017). Furthermore, in polyandrous species; females mate with multiple partners leading to intense competition between males where the latters need to develop effective strategies to attract females and ensure reproductive success (Parker & Birkhead 2013; Morimoto et al. 2019). Polyandrous females generally prefer males with traits that indicate higher reproductive quality, as multiple mating may be related to obtaining additional resources from males such as nutrients (Ward & Landolt 1995; Arnqvist & Nilsson 2000).

The tomato pinworm *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae) is a destructive pest on tomato crops that has invaded more than 90 countries (Biondi et al. 2018; Mansour et al., 2018; Han et al. 2019; Desneux et al., 2022). Its control has been traditionally based on pesticides applications but significant technological advances have been made in the last decades allowing integrated management of this pest. Thus, synthetic sex pheromone--based strategies are actually used in field monitoring efforts, mass trapping and mating disruption for *T. absoluta*. However, experiments using synthetic sex pheromones to control *T. absoluta*, reported that despite the high suppression rate of males in the field, losses in tomato production were still above the economic damage level (Lôbo 2005; Jallow et al. 2020). These failures may be linked to the polyandrous sexual behavior of this species, as well as migration or competition between females and traps at high population densities (Lôbo 2005; Jallow et al. 2020). Knowing that female insects prefer mating with individuals with higher reproductive quality

(Marcotte et al. 2007; Muller et al. 2016) and that an insect's reproductive quality can vary depending on factors such as body size and weight, mating status, age and sperm quantity and quality (Kelly & Jennions 2011; Xu & Wang 2020), we hypothesized that *T. absoluta* females attract and choose their male partners based on their higher reproductive quality (heavy, young, virgin), while rejecting males that may be considered of lower quality (light, old, mated). This leads the rejected males to be more attracted to traps containing synthetic sex pheromones.

Due to the lack of knowledge about the mechanisms underlying female choice and its impacts on the reproductive ability of the male of *T. absoluta*, it is important to understand the reproductive behavior of these insects in order to best interpret the results obtained in the field. In fact, previous studies with this species suggest that body weight and mating history may be related to the overall quality of males (Caballero-Mendieta & Cordero 2013; Muller et al. 2016). Heavier and virgin males can produce larger spermatophores, resulting in greater reproductive success (Lee et al. 2014; Lôbo 2015). Therefore, females seek advantages from these males, such as increased fecundity, fertility and greater offspring success (Lingren et al. 1988; Calvo & Molina 2005).

Therefore, the aim of this study is to evaluate how certain parameters of male reproductive quality, such as body weight, mating status and age, may influence the reproductive behavior of *T. absoluta* females, as well as their receptivity to these males.

Materials and Methods

1. Rearing of *Tuta absoluta*

The rearing and experiments were carried out at the Laboratory of Semiochemicals and Insect Behavior at the Federal University of Viçosa (UFV), MG, Brazil. Tomato plants *Solanum lycopersicum* (variety Vivaty) were used for both egg laying and larval feeding of *T. absoluta*. The seeds were sown in plastic trays filled with vegetable growing substrate (MecPlant R soil, MecPlant, Telêmaco Borba/PR-Brazil) and stored in a germination chamber (Tecnal TE-

4000/1). Subsequently, the seedlings with 2-3 leaves were transplanted into 3 liter pots filled with substrate conditioner and transferred to a greenhouse with a temperature of $25 \pm 2^\circ\text{C}$ and relative humidity of $70 \pm 5\%$. Macronutrients were supplied every 7 days with NPK fertilizer (10:10:10, Fertipar Group, Curitiba, PR-Brazil).

Tuta absoluta was reared in a breeding room with controlled conditions regarding temperature ($25 \pm 2^\circ\text{C}$), photoperiod (L12:D12), and relative humidity ($70 \pm 5\%$). The collected adults were placed in acrylic cages (30 x 30 x 30 cm) and fed ad libitum with a 10% sugar solution, and with tomato leaves for egg laying. Egg-laying plants were transferred every two days to wooden cages (50 x 40 x 40 cm) covered with organza fabric to allow air circulation. After hatching, the larvae were fed with tomato leaves kept in containers with water to prevent them from losing their plumpness. Following the larval period (15 to 20 days), pupae were removed from leaves and placed in petri dishes (90 x 15 mm) in cages for development and emergence of adults.

2. *T. absoluta* males' and females' weight groups' separation

The weight groups of *T. absoluta* males and females were established based on pupal weight, according to the protocol of Muller et al. (2016). Pupae aged 2 to 3 days were removed from tomato leaves and sexed based on the location of the genital pore (Coelho & França, 1987) and weighed on a SHIMADZU AUW220D analytical balance. Weight ranges were estimated using the weight of 200 pupae for each gender. The females used in the experiments had a pupal weight between 2.10 and 3.43 mg. The males' pupae were divided into three groups: light (weight between 1.33 and 1.87 mg), median (weight between 1.89 and 2.66 mg), and heavy (weight between 2.69 and 4.00 mg). Pupae of males (300) and females (300) of all weight groups were individualized in 70 mL plastic cages sealed with organza mesh and monitored until emergence.

3. *T. absoluta* males' and females' age and mating status groups' separation

Recently emerged males (300), were first divided according to their weight into the three weight groups described previously (i.e. light (100), medium (100), and heavy (100)); then divided according to their age into 150 young (three-day-old) and 150 old (six-day-old) males. The categorization of males according to their weights and age allowed the establishment of six groups of 50 males (young x light, medium, and heavy and old x light, medium and heavy).

Within the six groups, 25 males (24 hours old) were mated with a virgin and copulation was considered effective if it lasted at least 30 minutes. In preliminary observations, copulations averaged 202.50 ± 89.48 minutes in duration. This criterion of a minimum duration of half an hour for successful copulation was applied because males must have sufficient time to complete spermatophore transfer, a process that takes a long time (Ouye et al. 1965; Lee et al. 2014). The females served solely to change the males' mating status from virgin to mating. After a successful copulation, the females were returned to the breeding cages.

The males' separation by weight, age and mating status allowed the distinction and establishment of the following twelve male "categories" combining various male characteristics (Table.1).

4. Experiments

The experiments were carried out in two stages. In the first step, we examined female choice behavior to obtain general parameters of male reproductive quality. We then analyzed how these factors influenced female egg-laying, fecundity and fertility. The entire experiment was carried out in controlled rearing rooms with controlled photoperiod, temperature and relative humidity according to the rearing procedures described previously.

4.1. Effects of male weight, age, and mating status on the mating preference of *T. absoluta* females.

The choice behavior of *T. absoluta* female was examined in medium-sized, two-day-old virgin females. Females were housed in 70 mL plastic cages closed with organza mesh into

which two males from two different male categories were previously introduced. The following combinations of male categories were tested (see Fig.1): (A) young males: light vs. heavy, (B) old males: light vs. heavy, (C) young medium males: virgin vs. mated, (D) old medium males: virgin vs. mated, (E) virgin males: light vs. heavy, (F) mated males: light vs. heavy. Another combination; Presentation of two hypothetical extreme scenarios; (G) Young, virgin, and heavy males compared to old, mated, and light males were also tested.

The male-female pairs (see Fig.1) were placed in individual cages and monitored during the photophase, from 06:00 to 18:00. Copulation between the female and the chosen male was observed until its end (202.5 ± 89.48 minutes) when the insects separated, and then the choice was recorded. To be considered effective, copulation had to last at least 30 minutes (Ouye et al. 1965; Lee et al. 2014). For each comparison between a set of two males categories, 25 repetitions (one female and two males from two different categories) were performed.

4.2. Effect of combining the most distant male characteristics on egg laying, fertility, and longevity of *T. absoluta* females

Based on the results of bioassays of female choice behavior, two groups of males were standardized by weight, age, and mating status (as previously described). The first group combined the characteristics of males that were most rejected (i.e., old, light, and mated (n=30)), and the second group presented the characteristics of males that were most preferred by females (i.e., young, heavy and virgin (n=30)).

Each male was mated with a 2-day-old virgin female, and after mating, females were placed in 70 ml plastic cages closed with a two-leaf organza net and in a 1 ml glass jar with water for oviposition given. To be considered effective, copulation had to last at least 30 minutes and result in fertile eggs that later hatched into larvae. The number of eggs laid by each female was counted every two days, and the leaves were replaced accordingly until the female died. The glass jar containing the leaves and the laid eggs was kept in 2-liter plastic cages closed

with organza nets, and the number of hatched larvae was counted every two days until no more larvae hatched. For longevity analysis, the number of days from adult emergence to death was counted.

4.3. Influence of male weight on egg laying, fertility and longevity of females of *T. absoluta*

Based on the results of bioassays on female choice and its effects on the reproductive process, we standardized the weight factor using males of average weight, different ages and mating status to assess the influence of male weight effectively. The characteristics of the most rejected males, excluding the weight factor (i.e., middle, older, and mated type), were combined with those of the group that had the characteristics of the males most preferred by the females (i.e., middle value, young and virgin). The number of eggs laid by each female, the number of hatched larvae, and female's longevity were assessed as described in the previous bioassay.

5. Statistical analyses:

Statistical analysis were performed using R software (v. 4.0.0; R Development Core Team, 2020). Female choice, fecundity (number of eggs/female) and fertility (number of larvae/female hatched) were assessed for each experiment (Fig.1) using generalized linear models (GLMs); with binomial, Poisson and binomial error distributions, respectively; followed by pairwise comparisons of Least Squares Means (emmeans). The male's weight (light and heavy), male's status (mated and virgin) and male's age (young and old) were considered the fixed factors. Residual analyses were performed to validate the error distribution and model fit. To analyze longevity, statistical tests were performed using survival analysis, using the Weibull distribution model.

Results

1. Influence of combinations of male characteristics on the mating preference of *T. absoluta* females

1.1. Influence of male age and weight

When female have to choose between a male of different ages and weights, the results showed a significant (GLMs with Quasi-binomial: $n=25$, $F= 6.362$, $p < 0.001$) effect of interaction between these two attributes (age and weight) in the female's choice (Fig. 2A). Furthermore, when comparing female choice in the presence of light and heavy males, females consistently preferred (GLMs with Quasibi-nomial: $n = 25$, $F = 3.814$, $p < 0.05$) heavy males regardless of male age (whether young or old) (Fig. 2B and 2C).

1.2. Influence of age and mating status of males

Females' mating preference behavior was found to be independent of males' age but not of their mating status (Fig. 2D; GLM with Quasi-binomial family: $n = 25$, $F= 3.481$; $p < 0.001$). Whether the male was young (Fig. 2E; GLMs with Binomial family: $n = 25$, $F= 6.363$, $p = 0.01$) or old (Fig. 2F; GLM with Quasi-binomial: $n = 25$, $F = 3.481$; $p < 0.001$), females consistently preferred virgin males to mated males (Fig. 2 D, E, F).

1.3. Influence of weight and mating status of males

When testing the hypothesis that *T. absoluta* females prefer heavy virgin males to mated light ones, the results showed that heavy males were selected by females more often than light males, regardless of their mating status (GLM with Quasi-binomial family: $n = 25$, $F = 3.814$, $p < 0.005$) (Fig. 2 G, H, I).

1.4. Effect of combining the most distant male characteristics

Based on the previous results, female mate choice was tested in two highly contrasting situations by selecting males with the traits that elicited the highest preference (young, virgin and heavy) and lowest preference (old, mated and light) selection rates in the females. The results confirmed that a significantly higher number of mating occurred between females and young, virgin, and heavy males (GLMs with Quasi-binomial family: $n = 30$, $F= 9.545$, $p = 0.003$) (Fig. 3).

2. Influence of combinations of male characteristics on the egg laying, fecundity and longevity of *T. absoluta* females

2.1. Effects of male weight, age and mating status

The effects of male weight, age and mating status on females' egg laying, fertility and longevity were assessed by combining females with either young, virgin and heavy males or old, mated, and light males (Fig. 4 A, C, E). The results showed that no significant differences in the number of eggs (Fig. 4C; GLMs with Quasibi-nomial family: $n = 25$, $F = 3.150$, $p > 0.05$) laid by females mating with young, virgin and heavy males and females mated with old, mated, and light males. On the other hand, a higher number of larvae hatched from eggs laid by females that mated with old, mated and light males (Fig. 4E; GLMs with Quasi-binomial family: $n=25$, $F= 6.949$ $p < 0.001$).

Moreover, the longevity of females was significantly higher (survival analysis with Weibull distribution; $n = 30$, $\chi^2= 6.45$, $p < 0.001$) when they mated with the preferred males often selected being young, virgin, and heavy compared to females that mated with less preferred males (old, mated and light) (Fig. 4A). While the 50% population mortality (LT_{50}) was reached after 20.26 days in females mated with the less preferred male, it only occurred after 24.52 days for the more preferred males.

2.2. Effects of male age and mating status

Ignoring the weight variable, no significant difference was found when analyzing the number of eggs laid by females mating with young, virgin males compared to females mating with mated, old males (GLMs with Quasi-binomial: $n = 30$, $F = 2.007$, $p > 0.05$) (Fig. 4D). Likewise, no significant difference was observed between the two combinations when assessing fertility in relation to virgin, young males versus mated, light males, (GLMs with Quasi-binomial: $n = 30$, $F = 15.58$, $p > 0.05$) (Fig. 4F).

Furthermore, the mating of females with young, heavy males or old, mated males had not direct impact on the longevity of the females, and no significant difference was observed between the two groups of males (Survival analysis with Weibull distribution: $n = 30$, $\chi^2 = 0.03$, $df = 1$, $p > 0.05$) (Fig. 4B).

Discussion

Choosing the best partner is crucial to the dynamics of sexual selection (Darwin 1872). The mate choice in relation to reproductive costs has been extensively studied in various species (Liu et al. 2011; Jing et al. 2023) and the female's choice has been shown to be often influenced by the status of the males (Darwin 1872; Jing et al. 2023). When testing the mating preference of *T. absoluta* females, we found that the best conditions for reproductive success occurred when the male was young, virgin, and heavy, while females showed greater rejection toward old, mated, and light males. A similar choice mechanism has been previously documented in other insect species, such as in the wasp *Pachycrepoideus vindemmiae* (Hymenoptera: Pteromalidae) (RONDANI, 1875) (Jing et al. 2023).

Regarding the mating status of males, female preference for virgin males may be related to a conditional factor, as studies have shown that males suffer a loss of spermatophore nutrients and size after each mating (Lôbo 2005). Lee et al. (2014) observed direct benefits when *T. absoluta* females were paired with virgin males rather than with the same male each time. In their experiments, mating occurred throughout the female's reproductive lifespan. This suggests that the females' preference of virgin males could be beneficial in terms of survival, reproduction and genetic diversity, as is known in other Lepidoptera (Arnqvist & Nilsson 2000; Xu & Wang 2009). In males of *T. absoluta*, a decrease of approximately 40% in spermatophore volume produced by males from the first to the second mating has been reported (Lôbo, 2005). This could explain the shorter longevity of females that mated with already mated males in our

experiments, as nutrient reduction in less preferred males may be related to a mechanism of sperm modulation.

In this study, body weight was found to be the main trait associated with the overall quality of mating decisions in *T. absoluta*. According to Calvo & Molina (2005), and Hatala et al. (2018) body size or body weight is an indication of the quality of the insects and is related to the provision of their ejaculate. The results also showed that *T. absoluta* females prefer to mate with heavier males, regardless of mating status and age. This connection between body size and reproductive success is discussed in various studies and implies that both sexes have evolved strategies to optimize their reproductive advantages and minimize the costs of selecting reproductive partners (Calvo & Molina 2005; Xu & Wang 2009; 2020). Interestingly, our results showed that females who mated with larger males had a longer lifespan, while fertility was higher in females that mated with smaller males. These results confirm that the male choice may bring direct benefits to females, such as increased fecundity, fertility, and/or longevity, thereby providing better resources for themselves and their offspring (Kirkpatrick & Ryan 1991; Calvo & Molina 2005) and thus support the theory that sexual selection increases with increasing reproductive success of the partner (Kelly & Jennions 2011; Liu et al., 2011).

Furthermore, when females were allowed to mate with males of extremely contrasting conditions (young, virgin and heavy versus old, mated and light), the female's choice resulted in longer lifespans for females that preferred the high-quality males (young, virgin and heavy) and increased fertility in females that mated with less preferred males (old, mated and light). Considering that numerous male species have adaptations designed to optimize the success of their sperm (Parker 1984; Simmons & Shuker 2014), the first plausible explanation could be the trade-off system based on patterns of sperm allocation and/or resource allocation for reproduction (Boggs 1995). More favored males could transfer a greater amount of nutrients to females via spermatophores, improving female's nutrition and thus inducing a positive effect

on the females' longevity. Larger, younger and virgin males can deposit more nutrients that favor female survival, as shown for the moth *Ephestia kuehniella* (Xu & Wang 2020) and for the parasitoid *Pachycrepoideus vindemniae* (Jing et al.,2023). On the other hand, less preferred males may invest more in offspring production through some mechanism of sperm modulation (Parker 1984; Simmons & Shuker 2014) as less preferred males who have already mated are likely to prioritize increasing offspring number over investing nutrients to increase female longevity. The fact that we did not observe differences in egg-laying between females that mated with less or more preferred males could be due to *T. absoluta* females requiring fewer nutritional contributions from males or because there is no apparent difference in nutritional contributions between virgin and second-mating males.

A second explanation could be related to the maturation process and sperm volume of older individuals, which may change during their adulthood (Brits 1979; Marcotte et al. 2007). Delayed mating in males can have a significant impact on the number of sperm transferred to females and potentially lead to physiological changes in the male (Brits 1979; Marcotte et al. 2007). These changes can lead to alterations in reproductive outputs such as fecundity and fertility or in females' reproductive behavior (reduced receptivity) (Brits 1979; Lingren et al. 1988; Marcotte et al. 2007). Similar changes have been observed in males of other members of the Gelechiidae family, particularly in *Phthorimaea operculella* (Zeller). In this species, the males can produce sperm throughout their lifespan, but the highest number of mature sperm in their testes was found to occur between the fifth and seventh days of their life (Brits 1979). This physiological change may be related to the higher fertility in *T. absoluta* females that mated with males from the less preferred group, which includes older males (6 days).

It is worth noting that *T. absoluta* exhibits polyandrous mating behavior (Lee et al. 2014), meaning that it would be beneficial for males to evolve mechanisms to achieve greater postcopulatory reproductive success in females mating with multiple partners to achieve and

consequently ensure their offspring (Simmons & Shuker 2014). Previous studies suggest that increased polyandry reduces pre-copulatory sexual selection on male mating success and promotes post-copulatory sexual selection (Parker & Birkhead 2013; Morimoto et al. 2019). For example, the male of this species may provide the female with a larger spermatophore or one with a thicker envelope in the female to activate the stretch receptor in the bursa, which is responsible for a behavioral change from acceptance to mating refusal (Smith 1984; Sugawara 1979). Xu & Wang (2020) examined the influence of weight on polyandry in a moth species and observed that females that first mated with lighter males were more likely to mate again than those that mated with heavier males to increase the ejaculate substitute. In a recent study of another moth species, Thorburn et al. (2018) showed that a female's likelihood of re-mating decreased as the number of sperm received in her reproductive tract increased.

Considering that females of *T. absoluta* receive more than enough sperm to fertilize their eggs in a single mating, regardless of the male (Xu & Wang 2009), polyandry may be more closely linked to the genetic benefit hypothesis, as it is the most likely scenario (Parker & Birkhead 2013). Because body weight is a heritable trait in moths (Xu & Wang 2009; 2020) larger males are more likely to mate with larger females, and both sexes benefit from genetic advantages associated with their sizes (Jiang et al. 2013; McDonald & Pizzari 2018).

However, in polyandrous species in nature, it is much more likely that individuals are often able to mate at least once (Arnqvist & Nilsson 2000; Parker & Birkhead 2013). To elucidate the effects of polyandry in *T. absoluta*, it is necessary to compare mating patterns in natural populations. It is important to note that there is a gap in studies on polyandrous patterns, investment and sperm modulation in these insects and the mechanisms controlling this choice pattern are poorly understood, highlighting the need for further research in this area to elucidate the reproduction of these insects underlies.

In summary, our results support the hypothesis that *T. absoluta* females have a mate selection mechanism based primarily on males' body weight and this can directly reflect on the female's reproductive quality. These findings are of great importance not only for understanding the reproduction of these insects but also for their practical implications in developing effective strategies to control agricultural pests such as *T. absoluta*. Based on the effects of mate choice, we suggested that, despite the high suppression rate of males, the observed control deficiencies in the fields where mass trapping is used as a primary might be related to female preference. Such behavior results in a larger number of only rejected males being caught in the pheromone traps but without effectively reducing the actual mating occurrence and, hence, without any significant effect on the pest population in the field.

Another practical implication could be within strategies using the male sterilization approaches for pest insect control (Simmons & Shuker 2014; Simmons et al. 2021). Continuous efforts are currently underway to improve the sterilization of *T. absoluta* males for use in field (Cagnotti et al. 2016; Kuyulu & Genç 2016; Paladino et al. 2016). Understanding females' preferences and their male choice behavior, may allow a selective release of more attractive sterile males that would lead to a higher competitive mating rate and, therefore, a reduction in the field population. Combined with other control techniques, this approach could effectively reduce the reproductive success and population size of *T. absoluta* in the field.

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Figure Legends

Figure 1: Experimental design describing the twelve male “categories” combining age (old and young), weight (heavy and light) and mating status (virgin and mated) characteristics of *Tuta absoluta* males used to test the female mating preference.

Figure 2: Mating preference of *Tuta absoluta* females in relation to the interaction of males’ age and weight (A, B, and C), of males’ age and mating status (D, E and F) and to males’ weight only. A: effect of the male weight (heavy or light) regardless of the age. B: effects of weight within the young males group. C: effects of weight within old males group. D: mating status (virgin or mated) regardless of the males’ age. E: mating status within young males group. F: mating status within old males group. G: weight independent of mating status. H: weight within virgin males group. I: weight within the mated males group. P values < 0.05 indicate statistical differences (GLM; Quasi-binomial family).

Figure 3: Mating preference of *T. absoluta* females toward males with two extremely contrasting traits (young, virgin and heavy vs. old, mated and light). P values < 0.05 indicate statistical differences (GLM; Quasi-binomial family).

Figure 4: Longevity of *Tuta absoluta* females when mated with either young, virgin, and heavy males or old, mated, and light (A) and when mated with either young and virgin males or light and mated males (B). P values < 0.05 indicate statistical differences between LT_{50S} (Survival analysis and Weibull distribution). The number of eggs (C and D) and the larvae hatching

proportion (E and F) of females when mated with either young, virgin and heavy males or old, mated and light males (C and E) and when mated with either young and virgin males or light and mated males (D and F). P values < 0.05 indicate statistical differences (GLM; Quasi-binomial family).

Table 1: Male categories resulting from combinations of various male characteristics.

		Age			
		Young (3 days)		Old (6 days)	
Weight	Light (1.33-1.87 mg)	Virgin 1	Mated 2	Virgin 3	Mated 4
	Median (1.89-2.66 mg)	Virgin 5	Mated 6	Virgin 7	Mated 8
	Heavy (2.69-4.00 mg)	Virgin 9	Mated 10	Virgin 11	Mated 12

1. young (3 days), light, and virgin
2. young (3 days), light, and mated
3. old (6 days), light, and virgin
4. old (6 days), light, and mated
5. young (3 days), medium-sized, and virgin
6. young (3 days), medium-sized, and mated
7. old (6 days), medium-sized, and virgin
8. old (6 days), medium-sized, and mated
9. young (3 days), heavy, and virgin
10. young (3 days), heavy, and mated
11. old (6 days), heavy, and virgin
12. old (6 days), heavy, and mated

Figures

Figure1:

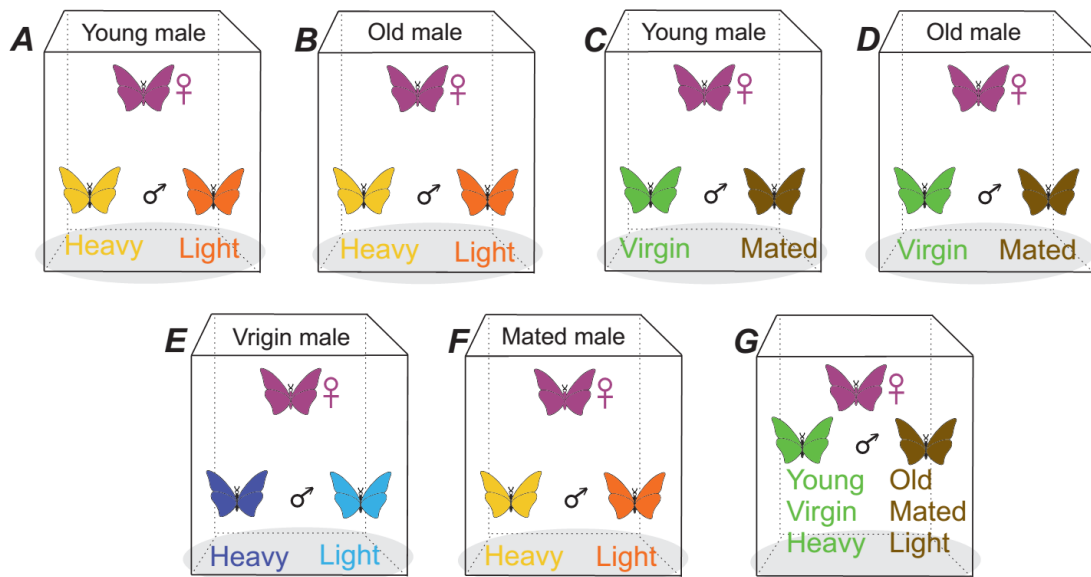


Figure 2:

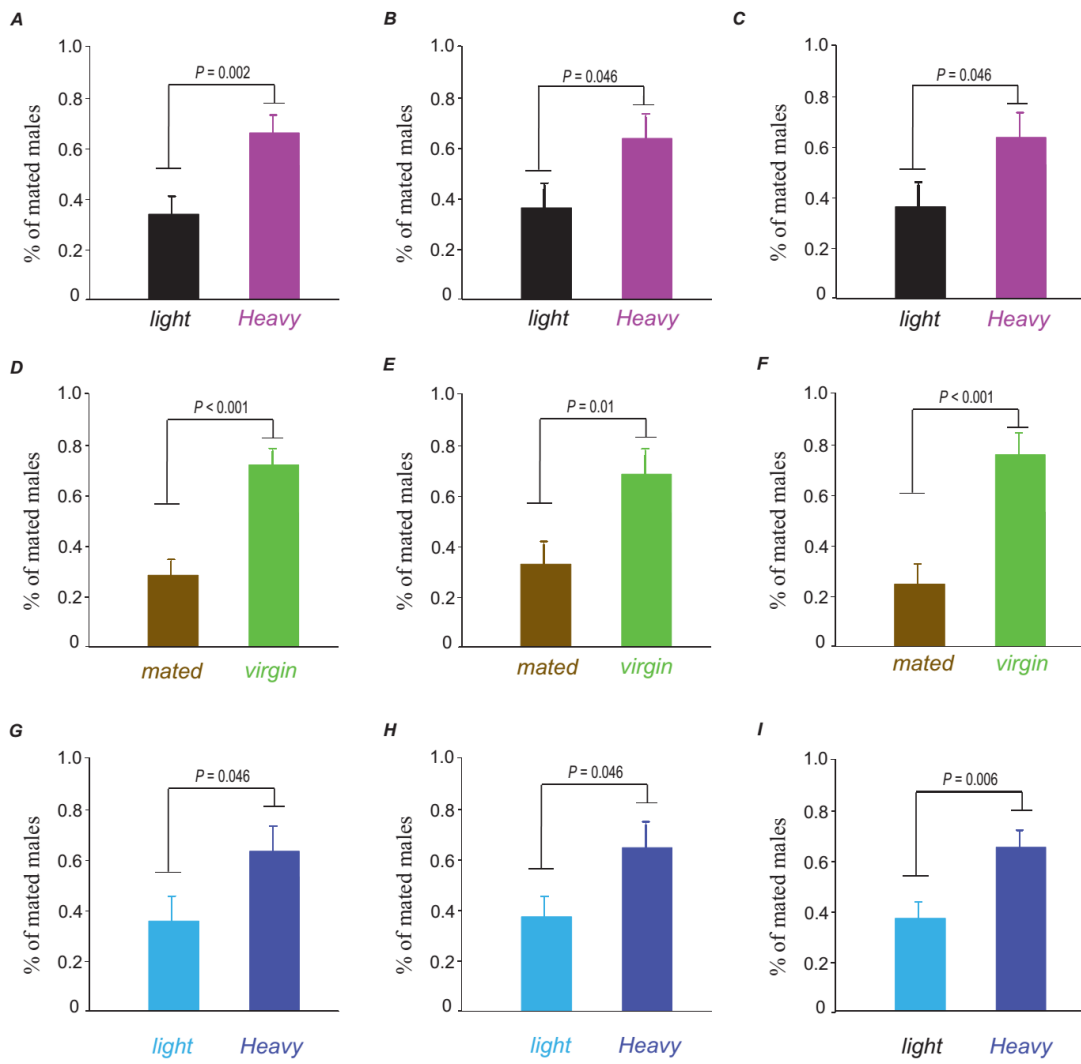


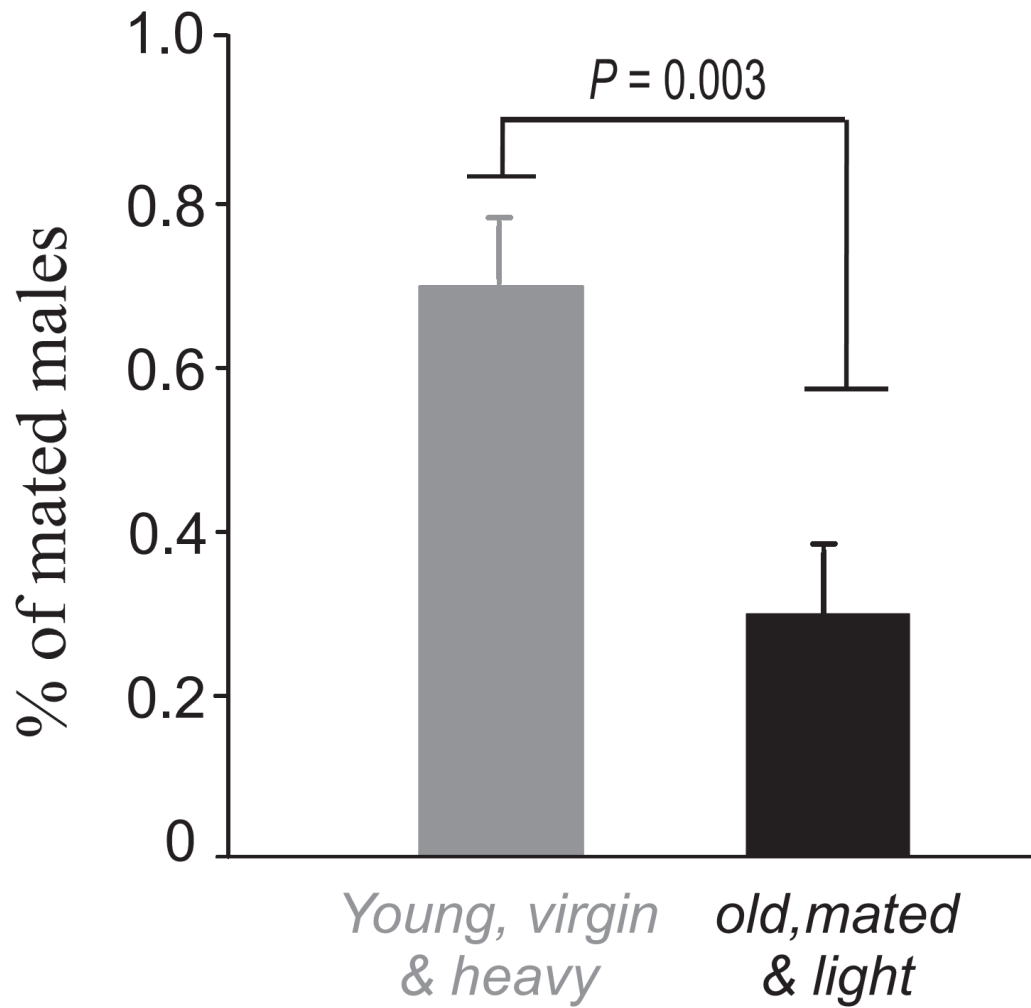
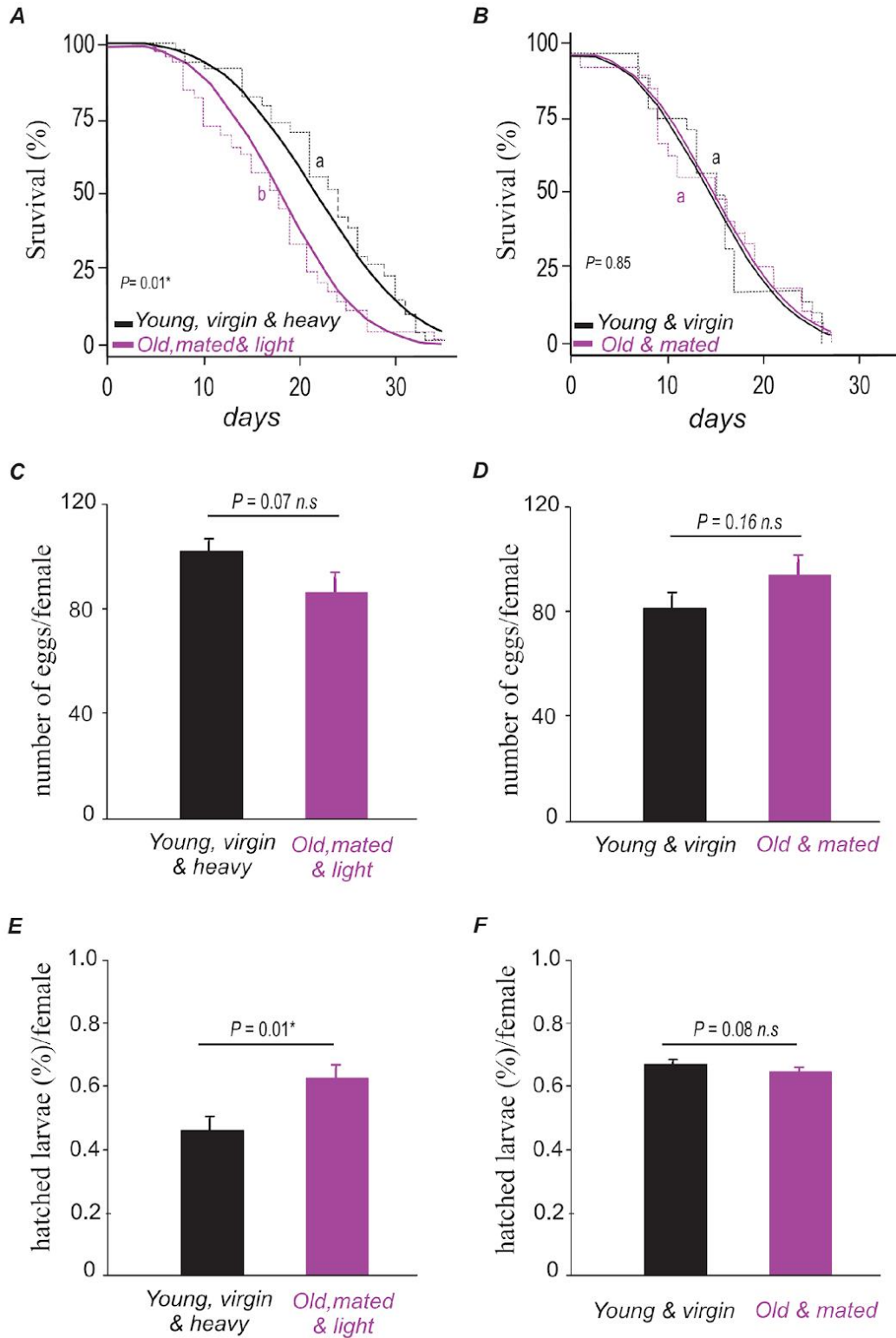
Figure 3:

Figure 4:



ARTICLE II

Body size effects on sperm quality of males of the tomato leafminer *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae)

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Abstract

The reproductive quality of an insect can vary due to biotic factors such as size, body weight, mating status, age, and quantity/quality of sperm. Males of *Tuta absoluta* can produce both eupyrene (nucleated) and apyrene (anucleated) spermatozoa types. Previous studies on *T. absoluta*, a polyandrous species, suggested a potential association between male body weight and reproductive quality. Heavier and virgin males were linked to larger spermatophores, indicative of higher reproductive success. It is anticipated that virgin and larger males contribute more to fecundity and fertility by transferring nutritional reserves through the spermatophore, presenting benefits sought by females for increased reproductive success. This study aimed to assess the sperm quality of light and heavy *T. absoluta* males. Testing the hypothesis that heavier males have better sperm quality, flow cytometry was employed to analyze the total quantity and viability of eupyrene sperm transferred to females mated with light and heavy males. Results indicated no significant difference in both the total quantity and viability of eupyrene sperm in spermatophores transferred to females, thus rejecting our hypothesis. However, a greater number of inviable spermatozoa were observed compared to viable ones in the copulatory pouch of females mated with both light and heavy males. Interestingly, when comparing field-collected individuals and the ones reared under controlled environments, a higher proportion of eupyrene sperm was observed in individuals from the natural habitat. This underscores the influence of the environment on the sperm quality of individuals. Consequently, the observed results suggest that the quantity, quality, and viability of sperm are not associated with the weight characteristic of *T. absoluta* males. Based on these findings, we propose that, in *T. absoluta*, male weight does not correlate with sperm quality.

Keywords: Sperm, viability, reproductive success, leafminer

Introduction

Sexual selection plays a fundamental role in the evolution of secondary sexual characteristics in many species, shaping the diversity of traits that males exhibit to attract mates (Arnqvist & Rowe, 2005; Parker, 1993; Birkhead et al., 2008). Additionally, reproductive quality and sperm competition are essential topics in the reproductive biology of many species, playing an important role in understanding the mechanisms underlying reproductive success (Arnqvist & Rowe, 2005; Simmons & Fitzpatrick, 2019).

As discussed by various authors (Parker, 1993; Ball and Parker, 1996; Simmons and Parker, 1992; Morrow & Gage, 2000; Snook, 2005; Birkhead et al., 2008), male body size also plays a central role in pre-copulatory and post-copulatory selection, and many studies have highlighted the positive correlation between body size and reproductive success. However, the relationship between body size and reproductive quality can be complex, as it also involves aspects related to phenotypic and genotypic quality (Parker, 1993; Ball & Parker, 1996; Johnstone, 1995; Birkhead et al., 2008). Studies on *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae), a polyandrous species, indicate that body weight and virginity may be characteristics associated with the overall quality of males. These studies showed that heavier and virgin males produced larger spermatophores, which may reflect in greater reproductive success (Lôbo, 2005; Lee et al., 2014; Wang, 2021; Gonçalves et al., 2024).

The tomato leafminer (*T. absoluta*), widely distributed in various parts of the world, poses a significant challenge in terms of control, particularly due to the escalating resistance to insecticides (Biondi et al., 2018; Desneux et al., 2010; Cocco et al., 2012; Guedes & Picanço, 2012). Strategies such as pheromone trapping and mating disruption, have been explored as management measures for dealing with this pest (Michereff Filho et al., 2000; Wang, 2021). However, the outcomes of these techniques continue to reveal considerable damage to tomato crops (Guedes & Picanço, 2012; Michereff Filho et al., 2000; Caparros et al., 2013; Wang et

al., 2021). A plausible explanation for these results may be linked to the polyandric reproductive behavior of the species and its spermathecal characteristics, such as sperm morphology and sperm viability, areas still underexplored in previous studies.

On the other hand, sperm competition, as addressed by Parker (1993), García-Gonzalez and Simmons (2005), and Birkhead et al. (2008), is a fundamental component of reproductive biology. In this context, sperm size, viability, motility, and speed of locomotion are attributes that play an important role (Radwan 1996; Hunter & Birkhead 2002; Simmons et al., 2003). Additionally, the quantity of transferred sperm is also a relevant factor in sperm competition (Parker & Pizzari, 2010). In Lepidoptera, sperm production is dichotomous, generating eupyrene sperm with a nucleus and apyrene sperm without a nucleus (Friedländer, 1997; Swallow & Wilkinson, 2002; Alves, 2006). Recent studies suggest the involvement of apyrene cells in sperm competition (Marcotte et al., 2005; 2007). Although not directly related to fertilization, apyrene sperm can represent a substantial proportion of the total of the two types (Friedländer, 1997; Swallow & Wilkinson, 2002). Sperm quality, including the ratio of eupyrene to apyrene sperm, along with the presence of nutritive substances, plays a fundamental role in determining reproductive efficiency and sperm competition in this species (Friedländer, 1997; Simmons, 2001; Snook, 2005; Swallow & Wilkinson, 2002).

Another relevant aspect is related to the viability of the sperm content in these males. Hunter and Birkhead (2002) suggested that males of insect species with polyandrous females tend to produce higher-quality sperm than males of monandrous species, as do other authors (Parker 1993; Birkhead et al., 2008; Fitzpatrick & Baer, 2011; Hunter & Birkhead 2002). However, it is crucial to note that, so far, there is no evidence supporting the idea that females are actively selecting males based on their body size in relation to sperm quality in *T. absoluta*. Therefore, the correlation between body size and reproductive success, while suggestive, still

lacks definitive evidence regarding its direct relationship with sperm adaptation. Thus, this study aims to analyze the viability and quality, both quantitatively and qualitatively, of the ejaculate from heavy and light *T. absoluta* males, as weight is the main characteristic in terms of reproductive quality in this species (Lôbo, 2005; Lee et al., 2014; Gonçalves et al., 2024).

Material and Methods

1. Rearing and field collection of *Tuta absoluta*.

The *T. absoluta* adults used in the experiments were obtained from either laboratory rearing or collected from tomato, *Solanum lycopersicum*, crop fields.

The laboratory rearing of *T. absoluta* was carried out in acrylic cages (30 x 30 x 30 cm) under controlled temperature (25 ± 2 °C), relative humidity ($70 \pm 5\%$), and photoperiod (12:12). Adults were provided, *ad libitum*, with 10% sugar solution, along with tomato leaves for oviposition. Plants, a variety Vivaty, used for egg laying were transferred every two days to wooden cages (50 x 40 x 40 cm) covered with organza fabric to facilitate air circulation. Upon hatching, larvae were fed with tomato leaves (2- 3 leaves) and placed in containers with water to prevent dehydration. After 15 to 20 days (larval stage), pupae were carefully removed from the leaves and placed in Petri dishes (90 x 15 mm) within cages for new adults' emergence.

The field-derived individuals were from an active collection that was conducted in the Baixo Russo crop, Coimbra, Minas Gerais, Brazil (-20.846781; -42.790910), targeting leaves containing larvae in the 3rd and 4th instar stages. These leaves were transported to and maintained in the laboratory until the larvae reached the pupal stage. Subsequently, the pupae were counted, sexed, weighed, and individually placed in 70 ml containers. The adults emerging from this active collection were used in the experiments.

2. Weights categories establishment for males and females of *Tuta absoluta*

To assess the effect of male body weight on reproductive quality, both males and females were grouped into different weight categories. The adults' groups were assigned using the pupal weight as described by Muller et al. (2016). In brief, 2 to 3-day pupae were carefully extracted from tomato leaves, sexed based on the genital pore's location (Coelho & França, 1987), and subsequently weighed using a SHIMADZU AUW220D analytical balance. Initial weight ranges were determined based on the weights of 200 pupae for each gender. The females used in the experiments ranged from 2.10 to 3.43 mg (lab), based on their pupae weight. The males were sorted into one of the four weight groups: light lab (weight ranging from 1.33 to 1.87 mg) and heavy lab (weight ranging from 2.69 to 4.00 mg), light field (weight ranging from 1.8 to 2.1 mg), and heavy field (weight ranging from 3.4 to 4.5 mg). After group sorting, all the pupae (300 males and 300 females) were individually placed in 70 mL plastic cages sealed with organza mesh and carefully monitored until their emergence.

3. Effect of male body weight on the testes size

Ten light (1.33 - 1.87 mg) and ten heavy (2.69 to 4.00 mg) virgin males of *T. absoluta* from laboratory rearing were dissected and the size of their testes was compared. Virgin males; 15 to 18 hours after emergence; to be dissected were initially anesthetized at -10°C for 2 minutes. Dissection was carried out in a 0.1 M phosphate-buffered saline (PBS), pH 7.2, and started by making a precise incision in the penultimate abdominal segment allowing the extraction of the male's complete reproductive system. After extraction, the testes were placed in Eppendorf tubes containing a 2.5% glutaraldehyde fixative solution. Subsequently, each testis was placed in Petri dishes with 2.5% glutaraldehyde fixative solution and photographed dorsally and ventrally using a Leica camera (MC170 HD) coupled to a Leica stereomicroscope (M205 A). After positioning the testes, at least ten photos of each testis were stacked to obtain better resolution and depth of the organs. The recorded photographs of the testes were then used

to measure the testes height, width, and circumference, with ImageJ software (Figure 1). Considering that the testis has an elliptical shape, the measured height, width, and circumference allowed to compute the testis surface area using the following formula :

$$\text{Surface Area (A)} = 4 * \pi * h * b$$

Where:

A: Represents the surface area of the testis.

π : Is the mathematical constant approximately equal to 3.14.

"b" = L/2 and corresponds to the major semi-axis of the testis (half of the width).

"h" = H/2 refers to the minor semi-axis of the testis (half of the height).

4. Effect of male body weight on the sizes of eupyrene spermatozoa and its nucleus

To compare the size of eupyrene spermatozoa from light (n = 5) and heavy (n = 5) males originating from the laboratory, the length of 30 sperm bundles from each male was measured, totaling 300 sperm bundles. It is important to note that, in *T. absoluta*, eupyrene spermatozoa are organized in bundles with uniform sizes and we assumed that each cyst contained 256 spermatozoa, as described by Garvey et al. (2000). The sperm bundles obtained from the seminal vesicles were transferred to histological slides and, after air drying, were carefully washed for 1 minute in distilled water. Subsequently, the histological slides were stained with Giemsa (according to formulation) for 30 minutes. After staining, the histological slides were washed again in running water for 1 minute and allowed to dry at room temperature. After this procedure, the slides were analyzed and photographed using a Q-Color3 digital camera attached to the Olympus BX-60 microscope. The length of each bundle was measured using ImageJ software.

To compare the nuclei length of eupyrene spermatozoa, we extracted them directly from the female bursa copulatrix. This ensured that each spermatozoa nucleus was independently evaluated, providing a precise analysis of their size. For this purpose, two-day-old virgin females and males from the laboratory rearing were used. Females were coupled with either one light or heavy male and allowed to mate. Matings were observed from the start until the end and only the females from couples that mated at least 30 minutes were considered. Five mated females of each mating group were dissected, as previously described for the males. The pouch containing sperm from each female's spermatophore was ruptured with a needle to release the sperm, which was gently spread on a histological slide. After being stained with 0.2 µg/ml of 4,6-diamino-2-phenylindole (DAPI) in PBS, the histological slides were photographed with an Olympus BX-60 photomicroscope with a digital camera and equipped with an excitation filter BP 360-370 nm, and the sperm nuclei length were measured using ImageJ software.

5. Effect of male body weight on the viability of spermatophores transferred to the bursa copulatrix of *T. absoluta* females

The spermatophores quality (presence, number, and proportion of eupyrene and apyrene spermatozoa) was analyzed by flow cytometry following previously established protocols (Graham, 2001). The characteristics of the spermatozoa were analyzed by association to specific fluorescent markers. Briefly, the female's bursa copulatrix containing the spermatophore of the males was placed in an Eppendorf tube containing 100 µl of PBS buffer solution (pH 7.2) and five minutes before the analyses, 5 µl of propidium iodide was added to the tube and connected to the flow cytometer BD FACS Verse. In the flow cytometer, as the sperm cells flow, they are impacted by wavelength of this UV laser of ≈ 488 nanômetros (nm), causing the excitation of the dye associated with the DNA of the spermatozoa, resulting in the emission of fluorescent light. This signal is then captured by photomultiplier tubes as the cells

pass in an orderly manner. Subsequently, the computational system processes the information and classifies the cells based on the observed luminescence (Sharpe & Evans, 2009). The viability of spermatophores transferred to 2-day-old virgin females' bursa copulatrix during mating with nine light and nine heavy 2-day-old virgin males from laboratory rearing was assessed through flow cytometry.

Furthermore, the bursa copulatrix of twenty females randomly picked from the laboratory population were dissected and the presence or absence of sperm was analyzed to confirm the viability of males' sperm in the laboratory population.

6. Assessment of sperm quality from males of *T. absoluta* laboratory-reared and field-collected

Initially, the potential difference in sperm viability between lab-reared and field-collected males was assessed using 18 females that were allowed to mate with lab-reared (n=9) and field-collected males (n=9). The males were randomly chosen regardless of their weight. The quantity of apyrene spermatozoa was calculated by subtracting the number of eupyrene spermatozoa resulting from viability tests from the total number of spermatozoa. Subsequently, the effects of body weight on the quantity and proportion of each spermatozoa type were assessed. A total of 40 spermatophores were used. These spermatophores were extracted from females' bursa copulatrix after mating with either 20 light (n=10) and heavy males (n=10) from the field, or 20 light (n=10) and heavy males (n=10) from the laboratory rearing.

7. Statistic

The analyses were performed in the statistical software R (v. 4.0.0; R Development Core Team, 2020). The number, and viability of sperm and the presence of sperm content in the bursa copulatrix, were analyzed through generalized linear models (GLM) with quasipoisson error distribution, followed by pairwise comparisons of Least Squares Means (emmeans).

The evaluation of eupyrene spermatozoa size, and nucleus size were determined through generalized linear models (GLM) with Gaussian error distribution. Subsequently, residual analyses were conducted to confirm error distribution and model adequacy.

Additionally, we quantified the variation in sperm length by calculating the coefficient of variation ($CV = [\text{standard deviation}/\text{mean}] \times 100$) in the total length of sperm between light and heavy males. The CV was used as a standard measure of variance in sperm characteristics, as established in previous studies (Malo et al., 2006; Calhim et al., 2007).

Results

1. Reproductive anatomy and the effect of male body weight on the testes size

Male of *T. absoluta* exhibits a pair of fused elliptical testes, followed by two small vas deferens. Subsequently, at the beginning of these, two dilations are corresponding to the seminal vesicles and, later, the post-vesicular vas deferens. The testes are oval, and brownish, measuring an average of $0,396 \pm 0,027$ mm (Figure 2A). In sexually mature males, the testes contain apyrene (Figure 2C) and eupyrene (Figure 2D) spermatozoa bundles. The female's copulatory bursa has a pyriform shape, in the apical region, the most dilated area, and it has a translucent yellow homogeneous content. The lower part is tapered, where the sperm content is located (Figure 2B). Both eupyrene and apyrene spermatozoa dissociate shortly after passage into the spermatophore. Eupyrene spermatozoa, measure on average 400 ± 23.2 μm (Figure 2F). These are flagellated and filiform (Figure 2E, F).

The measurements of the testes were used to compare the reproductive quality of light and heavy males. However, we did not observe a significant difference, both in the area (GLMs with gaussian family: $n = 10$, $F = 0.7281$, $p = 0.4007$) and in the circumference (GLMs with gaussian family: $n = 10$, $F = 0.7412$, $p = 0.3966$) of the testes between the two groups. The testes area ranged from 0.396 ± 0.027 mm (heavy males) to 0.361 ± 0.030 mm (light males)

and the circumference ranged from 1.12 ± 0.04 mm (heavy males); $= 1.07 \pm 0.05$ mm (light males).

2. Effect of male body weight on the sizes of eupyrene spermatozoa and its nucleus

When comparing the influence of male weight on sperm size between heavy and light individuals, it was observed that heavy males exhibited significantly (GLMs with gaussian family: $n= 5$, $F= 4.0277$, $p = 0.004$) longer bundles of eupyrene spermatozoa (489.99 ± 2.32 μm) than the light males (483.37 ± 2.342 μm). A low coefficient of variation was observed for both heavy males' bundles (489.99 ± 48.73 , $CV = 0.0593$) and light males bundles (483.3 ± 28.55 , $CV = 0.580$). When analyzing the average size of the nuclei of eupyrenic sperm, it was noted that light males had sperm with significantly (GLMs with Gaussian family: $n= 5$, $F = 4.0083$, $p = 0.0462$) smaller nuclei (33.106 ± 0.0003513 μm) than heavy males (34.288 ± 0.047 μm) (Figure 6A and 6B). The same pattern of a low coefficient of variation can be observed in the nuclei of heavy spermatozoa (33.10 ± 5.854 , $CV=0.134$) and light spermatozoa (34.45 ± 39.36 , $CV = 0.169$).

3. Effect of male body weight on the deposition of spermatophores with or without sperm material transferred to the bursa copulatrix of *T. absoluta* females.

When comparing the quantity of filled spermatophores transferred to the bursa copulatrix of females between light and heavy males, it was observed that approximately 40% of the spermatophores transferred by light males did not contain spermatozoa, while in heavy males, this number was approximately 30%. There was no significant difference (GLMs with binomial family: $n= 10$, $F = 0.1428$, $p = 0.71$) in the total quantity of viable spermatophores between light males (0.56 ± 0.18) and heavy males (0.67 ± 0.17) (Figure 5A).

In addition to cytometry, we also analyzed to check for the presence of spermatozoa in a representative sample from the breeding. The results revealed that 75% of the females had

spermatozoa in the bursa copulatrix. Out of the 20 randomly evaluated females, 15 had spermatozoa in the bursa copulatrix, while 5 did not have spermatozoa in the bursa copulatrix (Figure 5B).

4. Assessment of sperm quality from laboratory-reared and field collected males of *T. absoluta*

To test the hypothesis that heavier males of *T. absoluta* have better sperm quality, parameters of quality were analyzed using flow cytometry for individuals from both field and laboratory rearing. Results indicated that, when analyzing weight factor in both groups of individuals (field and rearing), there was no significant difference in the numbers of both apyrene (GLMs with quasipoisson family: $n = 20$, $F = 1.60$, $p = 0.21$) (Figure 3A) and eupyrene spermatozoa (GLMs with quasipoisson family: $n = 20$, $F = 0.001$, $p = 0.97$) (Figure 3B), as well as in the total quantity of spermatozoa (Light = 812.0 ± 331.84 ; Heavy = 1053.3 ± 558.03) (Figure 3C) present in the spermatophores transferred to females (GLMs with quasipoisson family: $n = 20$, $F = 0.8716$, $p = 0.365$).

Similarly, the number of viability sperm transferred to the female's bursa copulatrix was not different (GLMs with quasipoisson family: $n = 9$, $F = 0.14$, $p = 0.71$) between light (26.00 ± 12.29) and heavy males (34.67 ± 18.41), in both field and laboratory-reared individuals (Figure 3D), indicating that weight does not influence the sperm quality of *T. absoluta* and that insects reared in the laboratory do not change concerning this parameter when compared to field insects. that the number of viable sperm

However, when comparing matings between males from the field and those reared in the laboratory, a significant difference was observed (GLMs with quasipoisson family: $n = 20$, $F = 17.48$, $p = 0.0001$) with field males (13667.30 ± 516.15) transferring a greater the number of viable of sperm to females compared to laboratory-reared males (13026.52 ± 455.48) (Figure

4A). The quantity of apyrene spermatozoa between field (10852.35 ± 177.43) and laboratory-reared males (10704.62 ± 147.15) did not differ ($p = xxx?$) (Figure 4B). However, when analyzing the quantity of eupyrene spermatozoa, field males presented a higher quantity (2814.95 ± 97.62788) compared to laboratory-reared ones (2321.91 ± 81.22) (GLMs with quasipoisson family: $n = 20$, $F = 20.5123$, $p < 0.001$) (Figure 4B).

Similarly, examining the viability of these eupyrene spermatozoa, we observed higher viability in females mating with field males (1253.26 ± 245.24) compared to those mating with laboratory males (704.5667 ± 235.52) (GLMs with quasipoisson family: $n = 9$, $F = 14.73$, $p = 0.0005$) (Figure 4C). However, it is important to note the significant increase in the number of non-viable spermatozoa in the bursa copulatrix of females, both for field males (1533.75 ± 279.72) and laboratory males (1630.0 ± 218.52) (Figure 4C).

Discussion

Male body size plays an important role in both pre-copulatory and post-copulatory selection, with various studies highlighting a positive correlation between *T. absoluta* body size and reproductive success (Simmons & Parker, 1992; Ponlawat & Harrington, 2007; Sturm, 2014; De Jesus & Reiskind, 2016). This relationship may be associated with phenotypic and genotypic quality (Johnstone, 1995). While research suggests a positive relationship between *T. absoluta* male body size and the quantity or quality of sperm transferred to females (Parker, 1993; García-Gonzalez & Simmons, 2005; Ponlawat & Harrington, 2007; Birkhead et al., 2008; Sturm, 2014), our data indicated that the quantity of sperm does not differ with the weight of males in *T. absoluta*. The opposite pattern was found by De Jesus and Reiskind (2016) studying sperm content in two *Aedes* species. It is noteworthy that, according to Parker and Pizzari (2010), the total number of sperm is the most evolutionarily influenced characteristic in sperm competition. However, the relative number of sperm does not explain all reproductive success (Birkhead & Møller 1992; Simmons et al., 2003).

It is worth noting that the interaction of the proportion between eupyrene and apyrene spermatozoa, along with the presence of nutritive substances, plays a fundamental role in determining reproductive efficiency and sperm competition in *T. absoluta* (Simmons, 2001; Snook, 2005). Among light and heavy *T. absoluta* males, this proportion did not vary, however, the higher quantity of eupyrene sperm from field individuals may be related to environmental processes and also to competition. Because more competitive males may have an advantage in transmitting their genes, in natural environments, this can be influenced by the proportion of eupyrene and apyrene sperm (Birkhead & Møller 1998;). Cohen (2018) emphasizes the importance of controlling and understanding environmental factors in rearing systems, as well as their influences on the genetic characteristics of insects bred in controlled environments.

The quality of sperm can be affected by various factors such as the size and quantity of sperm, viability, locomotion speed, and motility (Radwan, 1996; Hunter & Birkhead, 2002; Simmons et al., 2003; see Snook, 2005; Pitnick et al., 2009). When evaluating the viability of sperm transferred to females of *T. absoluta*, we did not find a correlation between viability and body weight. However, we observed, in general, a high sperm inviability, as we noted a higher number of dead sperm in the female copulatory pouch, which differs from the observations made by Hunter and Birkhead (2002), who found high sperm viability in some polyandrous insect species. Interestingly, in *T. absoluta*, a polyandrous species, we observed low viability in both light and heavy males.

García-Gonzalez and Simmons (2005) observed in *Teleogryllus oceanicus* (Le Guillou, 1841) that males with a higher proportion of live sperm in their ejaculates. In the case of *T. absoluta*, our results suggest that factors other than the males' body weight may be influencing sperm viability in this species. An additional explanation may be related to the passive loss of sperm, in which different proportions of the ejaculate reach the female tract at different times,

so that some sperm may die before any competition for fertilization occurs (Parker 1993; Ball and Parker 1996; Birkhead et al., 2008).

We observed in *T. absoluta* that the coefficient of variation showed low variability in the sizes of sperm bundles and nuclei. According to Fitzpatrick & Baer (2011), in some species of polyandrous ants and bees, post-copulatory sexual selection may result in a lower occurrence of errors during spermatogenesis, such as genetic mutations, DNA replication failures, or other events affecting sperm formation, as conceptualized by Hunter and Birkhead (2002). Therefore, in polyandrous scenarios, post-copulatory selective pressure may contribute to less variability in sperm (Calhim et al., 2007; Birkhead et al., 2008; Michalczyk et al., 2010; Fitzpatrick & Baer, 2011). Thus, it is plausible to suggest that males face a reproductive burden when producing more variable sperm.

However, it is known that sperm exhibit a wide range of characteristics affecting their fertilization capacity (Snook, 2005; Pitnick, 2009; Parker & Pizzari, 2010). In his theory on the relationship between sperm size and sperm competition, Parker (1993) highlighted that the evolutionary increase in sperm size might be related to their survival, but this connection can be ambiguous. When investigating the impact of insect weight on the size of eupyrene sperm in *T. absoluta* males, we observed that heavier males produce larger bundles of eupyrene sperm. This suggests a possible positive relationship between the relative length of the flagellum and increased motility, as emphasized by Casselman and Montgomerie (2004), Malo et al. (2006), and Birkhead et al. (2008), which could enhance reproductive success in terms of sperm competition in *T. absoluta*. According to Gomendio and Roldan (1991 and 2008), sperm competition and female choice may favor males with larger sperm, as they can provide competitive advantages such as higher speed, longevity, and replacement capacity, in addition to indicating the genetic quality of males to females (Parker, 1993; Ball & Parker, 1996).

Similarly, Morrow and Gage (2000) found a positive correlation between sperm size and sperm competition in butterflies and moths (see Snook, 2005).

However, it is important to consider that, as observed in various species, when sperm have longer flagella, the energy demands increase, leading to higher energy expenditure in situations of intense sperm competition (Gomendio & Roldan, 1991, 2008; Montoto et al., 2011). Similarly, the increase in sperm size in *T. absoluta* could result in reduced survival due to additional energy demands, particularly in an environment of intense sperm competition. Thus, for a definitive confirmation of this relationship and a more precise understanding, further research and specific experiments with this species would be necessary. A phylogenetic study found varied associations between sperm size and sperm competition in different taxa (Snook, 2005), which may also be related to *T. absoluta* males.

As heavy males of this species tend to present larger bundles of eupyrene sperm, the fact that nuclear eupyrene sperm from light males are larger could be an adaptation to effectively compete in an environment of intense sperm competition. This may be a resource maximization strategy to compensate for a possible disadvantage in terms of eupyrene sperm size. There is evidence that sperm competition can also impact sperm head morphology (Roldan et al., 1992). The impact of sperm size on fertilization competitive efficiency and the extent to which sperm size evolves in response to sperm competition seems to vary among taxonomic groups (Simmons 2001; Snook 2005; Montoto et al., 2011). In this context, evolutionary processes may have led light males of *T. absoluta* to strategic adaptations in the size of the nuclei of eupyrene sperm as part of a trade-off, possibly to compensate for the overall size disadvantage compared to heavier males (Parker, 1993; Parker et al., 2010).

In a study conducted with *Scathophaga stercoraria* (Diptera), Hosken & Ward (2001) observed that in situations of sperm competition, lineages subjected to forced monogamy

showed a lower relationship between testis size and body mass, as reported by Tomkins & Simmons (2002). This reduction in testis size may result from increased reproductive investment by smaller males adopting alternative strategies. However, in *T. absoluta*, no significant differences in testis size were found between light and heavy males. Kelly (2008) also found no differences in testis size in *Hemideina crassidens* (Orthoptera). Thus, it is evident that the exclusive evaluation of testis size in relation to individual weight is not sufficient for a comprehensive understanding of the complex dynamics of sperm competition and reproductive efficiency. The results of testis measurements may represent only one facet of reproductive quality in *T. absoluta*. Greenway et al. (2020) highlight that testis size is a highly variable characteristic, affected not only by male size but also by their body condition, such as physical state and mating opportunities.

Various authors (Parker, 1993; Simmons, 2001; Snook, 2005; Birkhead et al., 2008) emphasize that sperm quality, especially sperm competition, is a complex phenomenon and cannot be simplified into isolated numbers. Instead, to fully understand sperm competition, we need to take into account many factors, such as sperm quality, functionality, and strategies, along with environmental and behavioral influences affecting reproductive success (Parker, 1993; Simmons, 2001; Snook, 2005; Birkhead et al., 2008; Greenway et al., 2020). In this study, we explored various factors related to sperm quality in *T. absoluta*. However, it is important to recognize that complete understanding requires further investigations to unravel the complex interactions affecting reproductive quality in this species. Future studies can contribute to a comprehensive insight into this issue. In considering the resistance of the tomato leafminer to conventional strategies, this study contributes to advancing potential solutions by exploring a hitherto less-explored perspective. By deepening our understanding of the reproductive biology of this pest, it becomes feasible to direct efforts toward the development of more effective tactics tailored to its reproductive behavior.

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Figure Legends

Figure 1: Experimental Design for Testicular Measurements. L = width, H = height, T = semi-axis (1/2 height and 1/2 width), and t = circumference.

Figure 2: Reproductive feature of the adult *Tuta absoluta*. A = Male reproductive system. t - testis; dd - deferens duct; sv - seminal vesicle. B = Bursa copulatrix - bc. C = Apyrene sperm bundle - asb. D = Eupyrene sperm bundle - esb. E = Eupyrene spermatozoon: sptz. F = Nucleus of the eupyrene spermatozoon: f - flagellum, n - nucleus. Bars: Fig. A - 200 μm ; Figs. B - 2 mm; Fig C-F - 50 μm .

Figure 3: Sperm characteristics of ejaculate from light and heavy males in the field and laboratory rearing, transferred to the bursa copulatrix of *Tuta absoluta* females. A= Total number of spermatozoa; B= Total apyrene and eupyrene spermatozoa; C= Viability of eupyrene spermatozoa. NS = Non-significant difference ($p > 0.05$). The p-value was provided for the treatments [generalized linear model (quasipoisson family)].

Figure 4: Sperm characteristics of ejaculate from field and laboratory-reared males transferred to the bursa copulatrix of *Tuta absoluta* females. A= Total number of spermatozoa; B= Total apyrene and eupyrene spermatozoa; C= Viability of eupyrene spermatozoa. *** = Significant difference. Different letters indicate significant differences between the field and lab treatments ($p < 0.001$). The p-value was provided for the treatments [generalized linear model (family, quasipoisson)], followed by pairwise comparisons of Least Squares Means (emmeans).

Figure 5: Total viable spermatophores (with eupyrene spermatozoa) transferred to the bursa copulatrix of *Tuta absoluta* females. A= Total filled spermatophores between light and heavy males. B= Percentage of filled and empty spermatophores. NS = Non-significant difference at $p = 0.05$ for a generalized linear model (GLM) with binomial error distribution.

Figure 6: Comparison of Eupyrene Spermatozoa Size in Light and Heavy Males of *Tuta absoluta*. A= Mean size of eupyrene spermatozoa bundles (mm). B= Mean size of the nucleus (mm) of eupyrene spermatozoa. * = Significant difference ($p < 0.05$). The p-value was provided for the treatments [generalized linear model (family, gaussian)].

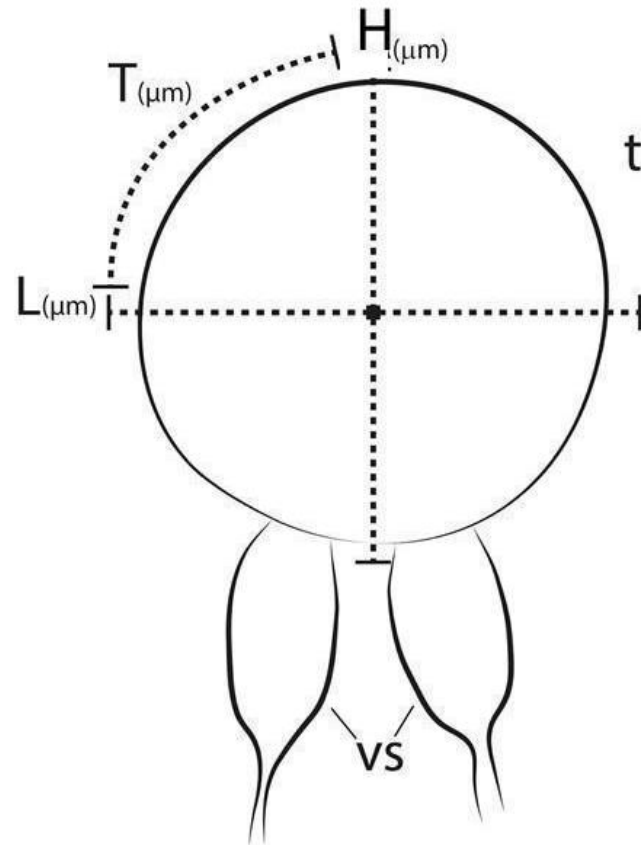
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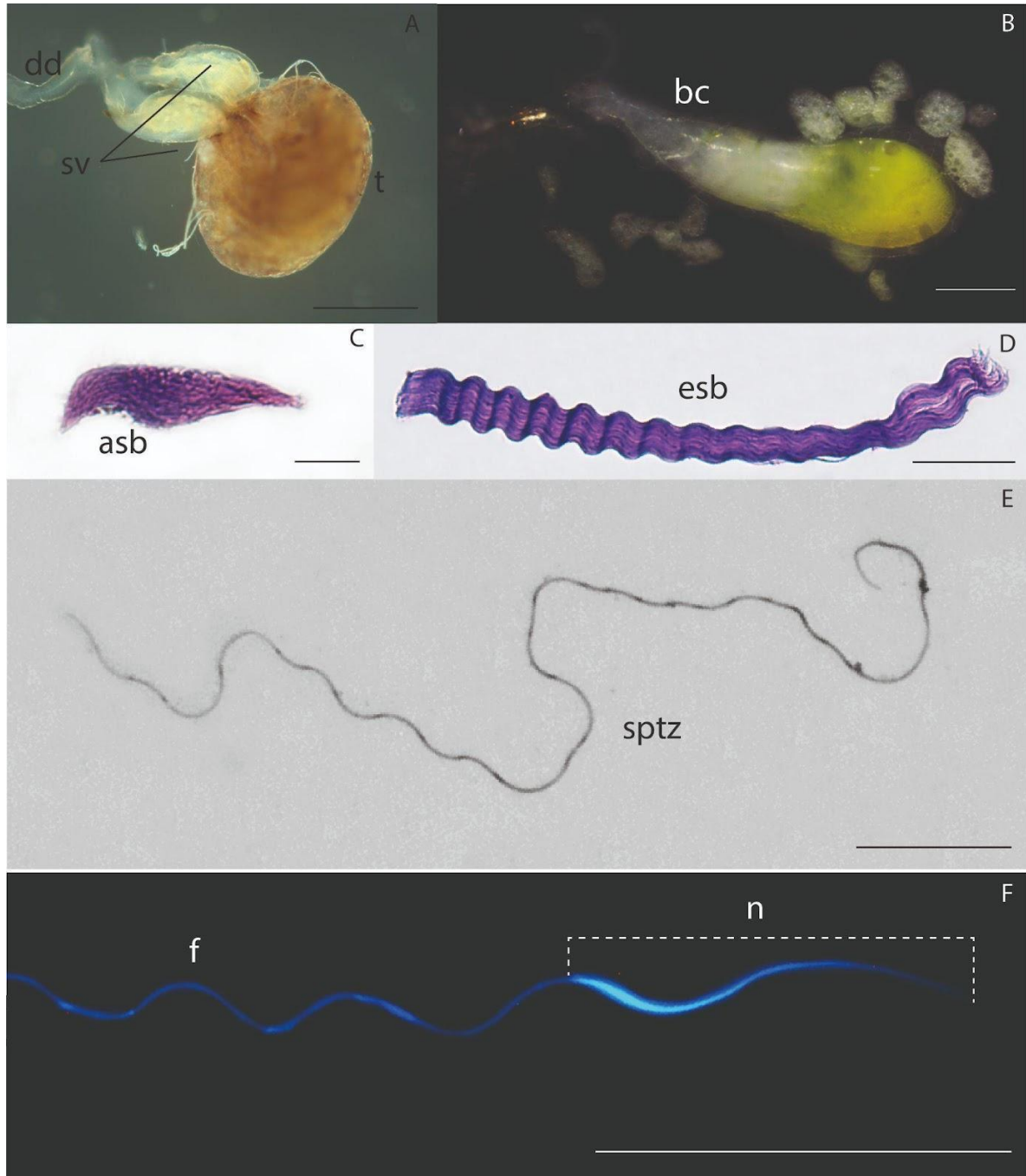
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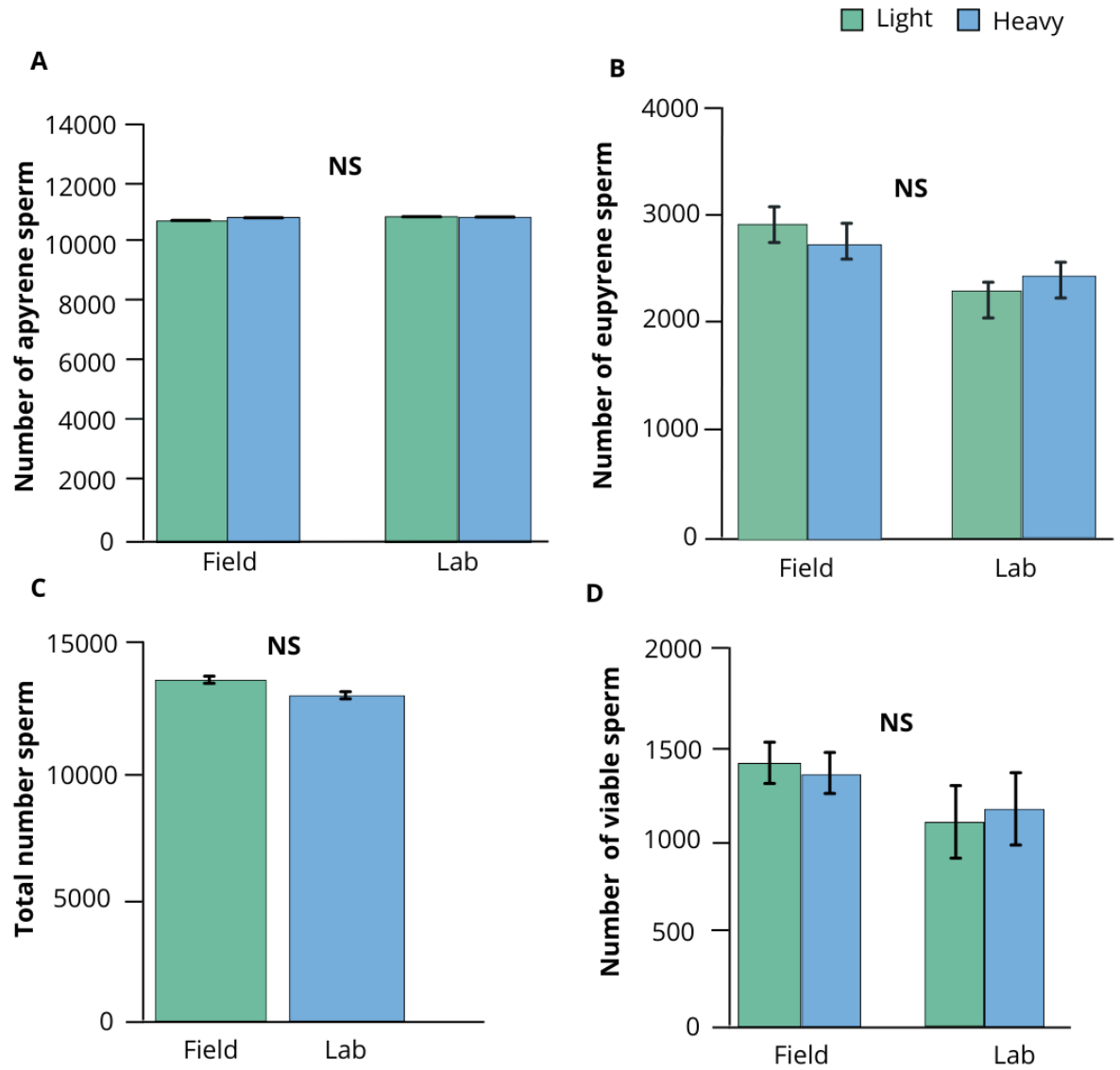


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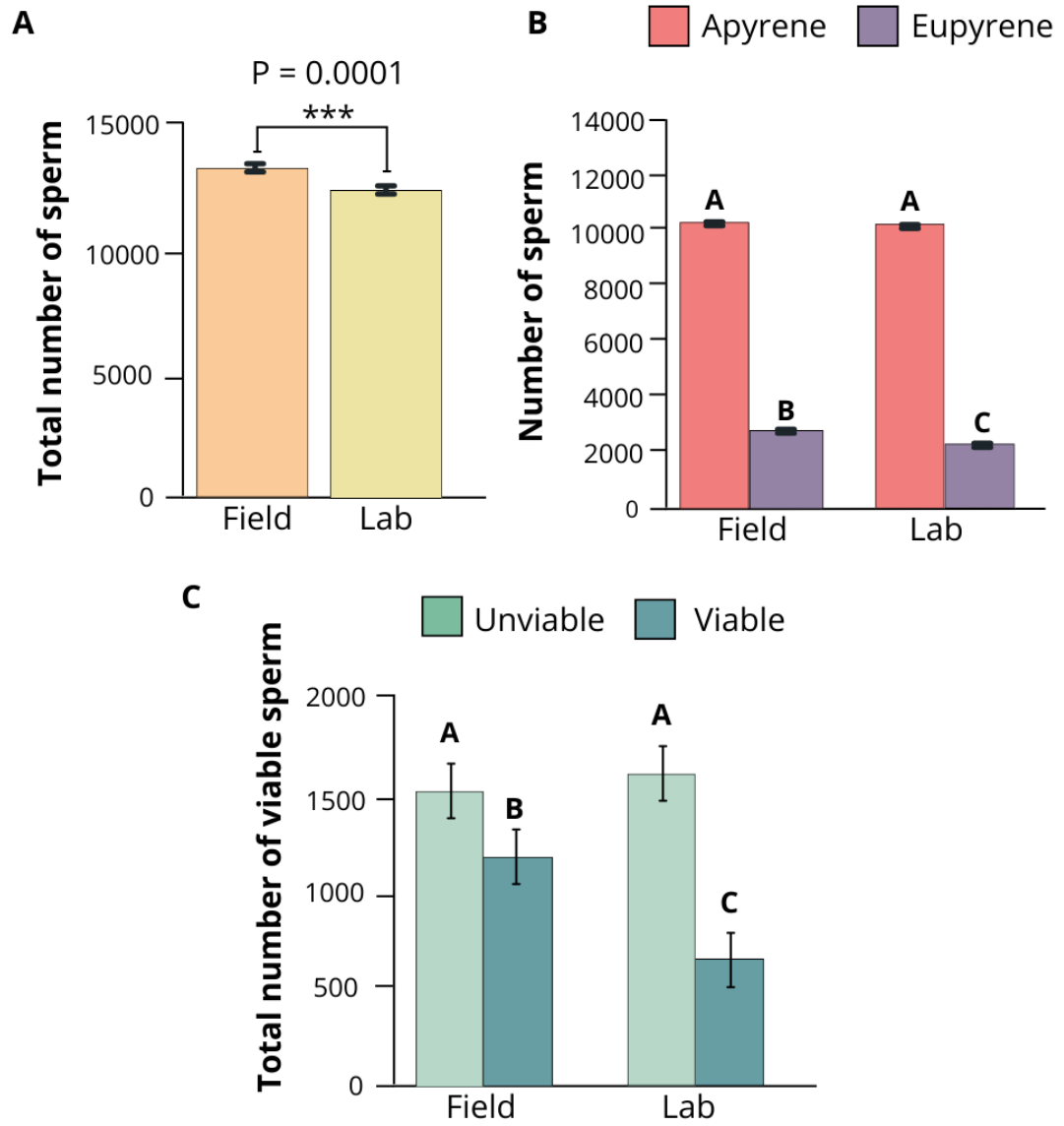


Figure 5:

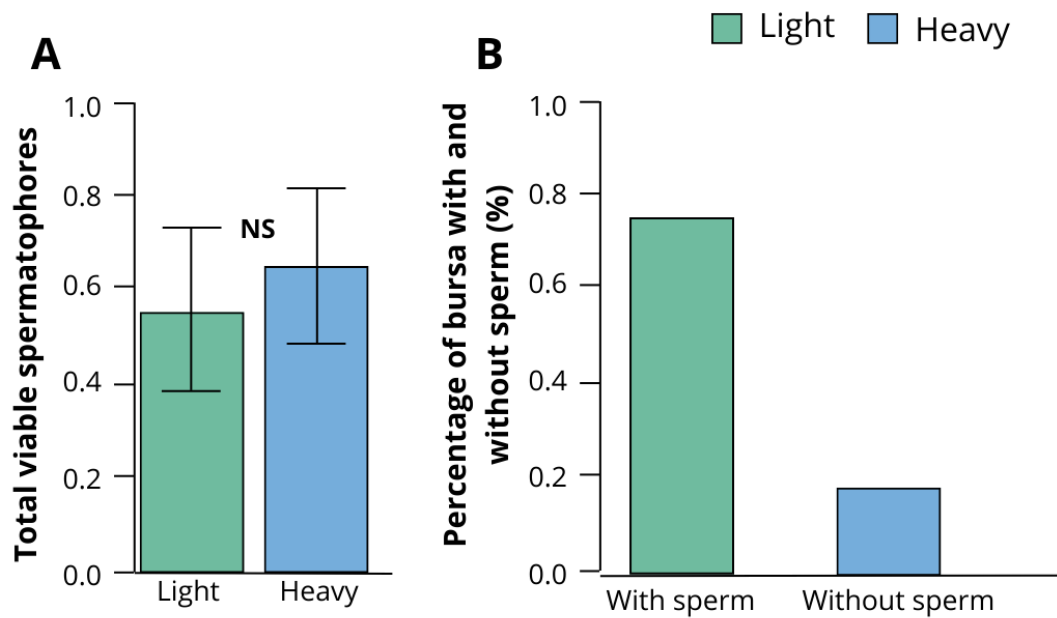
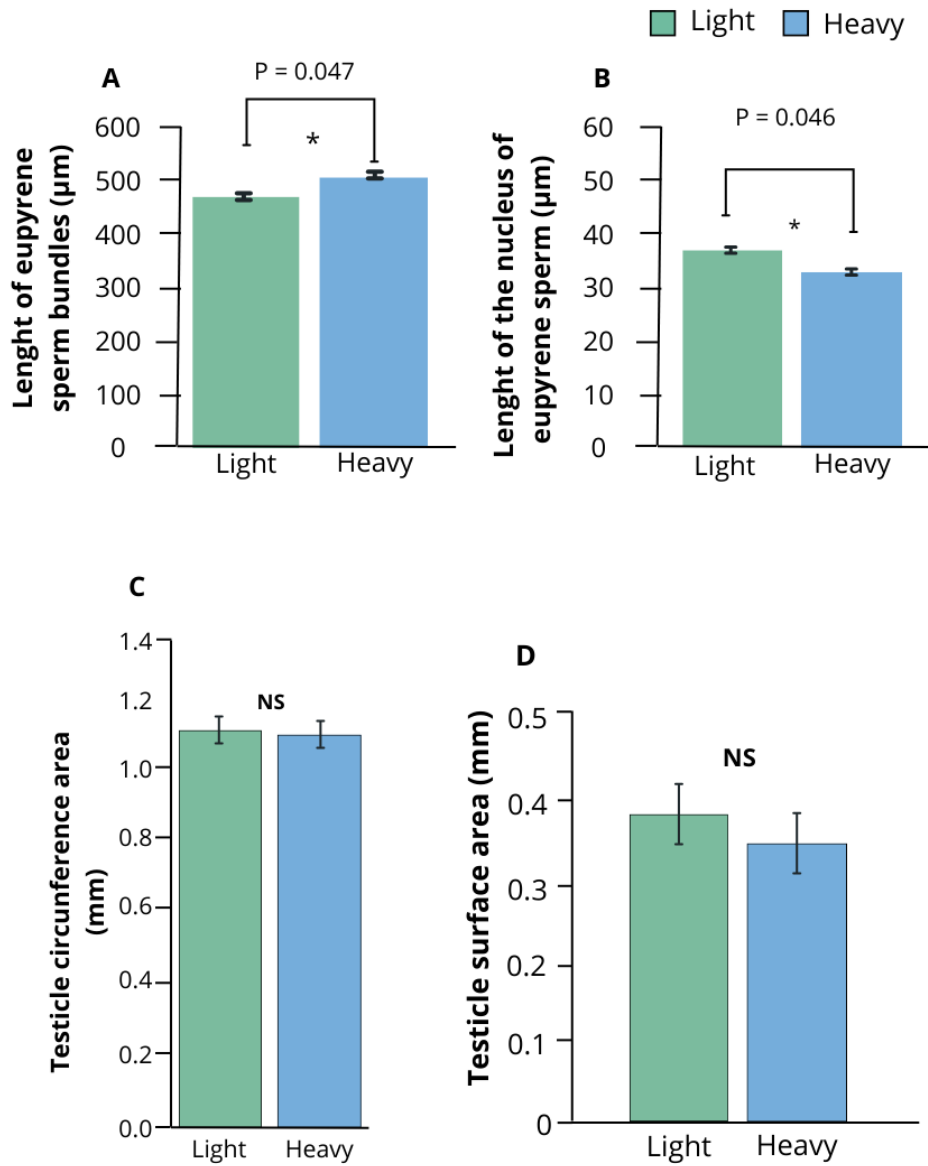


Figure 6:



ARTICLE III**Multiple Matings Do Not Impact Reproductive Parameters in *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae)**

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Abstract

The polyandry is a reproductive strategy that directly impacts the reproductive biology of insects, affecting female fecundity, longevity, and fertility, while also contributing to the genetic diversity of offspring. However, the direct effects of polyandry in *Tuta absoluta* have not been fully understood. Thus, the study aimed to assess the impact of multiple matings on the key reproductive characteristics of *T. absoluta*. For this purpose, females were subjected to different mating conditions, allowing a careful analysis of oviposition, fertility, and longevity parameters. The experiments were designed to isolate specific variables and standardize the number and timing of matings. Surprisingly, the results did not reveal significant differences in oviposition, fertility, and longevity between females who mated once and those subjected to multiple matings. This contrasts with some previous studies that highlighted the direct benefits of polyandry in other contexts. Our studies suggest that the effects of this mating strategy may manifest more subtly, possibly operating through indirect fitness, with genetic effects on offspring fitness. This study deepens the understanding of the reproductive biology of *T. absoluta* and highlights the complexity of these effects, emphasizing the importance of a careful approach to methodologies. The complexity of these effects underscores the importance of considering not only immediate results but also the long-term impacts on the population dynamics and evolution of this species, emphasizing the need for additional studies.

Keyword: Tomato leafminer, Polyandry, Oviposition, Fertility, Longevity

Introduction

The reproductive biology of insects is greatly affected by polyandry or the mating of a female with multiple males. This is because polyandry can directly increase a female's fecundity (the rate at which she oviposits), longevity, and fertility (the ratio between the number of larvae and the number of eggs) (Arnqvist and Nilsson, 2000; Parker & Birkhead, 2013; Gowaty, 2013; Li et al. 2014; Rafter et al.; Bocedi & Reid, 2017. 2018). The previously mentioned parameters primarily impact the reproductive life of females. It is known that three distinct factors affect all these components of fitness: the actual act of mating, the presence of sperm, and the transfer of accessory substances (Arnqvist and Nilsson, 2000; Gowaty, 2013; Parker & Birkhead, 2013). Indirectly, polyandry can increase the genetic diversity of offspring and the acquisition of good genes to enhance offspring fitness (Torres-Vila, 2013; Taylor et al., 2014; Li et al., 2014; Bocedi & Reid, 2017; Rafter et al., 2018). Providing a more genetically diversified offspring reduces the risk of inbreeding and adapts the species to variable environments, conferring greater ecological flexibility (Arnqvist & Nilsson, 2000; Jennions & Petrie, 2000; Tregenza & Wedell, 2002; Simmons, 2005; Gowaty, 2013). However, to date, the direct effects of multiple mating on female fitness have not been well defined (Arnqvist & Nilsson, 2000; Gowaty, 2013; Parker & Birkhead, 2013).

In polyandrous species, intense competition among males to attract females is an essential dynamic in reproductive strategy (Jennions & Petrie, 2000; Gowaty, 2013; Morimoto et al., 2019; Parker & Birkhead, 2013). This is observed in lepidopteran species such as *Tuta absoluta* (Meyrick, 1917), where it has been noted that polyandrous females play an active role in choosing their mates, preferring individuals of higher reproductive quality (Gonçalves et al., 2024). This selection behavior may be linked to the acquisition of supplementary resources by males, such as essential nutrients transferred through the spermatophore (Svard & McNeil, 1994; Simmons, 2001; Snook, 2005). Given that one mating is sufficient to fertilize every egg,

the acquisition of these nutrients may be linked to increased female longevity, which helps to explain why they frequently engage in multiple matings (Gonçalves et al., 2024; Xu & Wang, 2009, 2020).

Similarly, this may be related to the effort in controlling *T. absoluta*, globally recognized as one of the major tomato pests, causing substantial losses ranging from 80 to 100%, depending on the infestation level (Desneux et al., 2010). While traditional control involves the use of insecticides, integrated pest management has gained prominence, aiming to reduce the environmental impacts of excessive pesticide use (Cocco et al., 2012; Guedes & Picanço, 2012). The use of pheromone dispensers for mating disruption, despite being promoted as an effective technology, faces challenges evidenced by inconsistent results in controlling damage to the tomato crop (Michereff Filho et al., 2000; Wang et al., 2021). The observed lack of effectiveness may be linked to the reproductive behavior of the species and its polyandrous patterns, capturing already-mated males in traps (Michereff Filho et al., 2000; Lee et al., 2014; Gonçalves et al., 2024). Notwithstanding its promotion as a successful technology, the use of pheromone dispensers to disrupt mating poses specific difficulties, as shown by its uneven performance in reducing crop damage to tomatoes (Michereff Filho et al. 2000; Wang et al., 2021). Because of the species' polyandrous reproductive patterns and habit of trapping males who have already mated, the observed lack of effectiveness may be explained (Lee et al., 2014; Michereff Filho et al., 2000; Wang et al., 2021; Gonçalves et al., 2024).

The research conducted by Lee et al. (2014) on *T. absoluta* highlighted the direct benefits associated with multiple matings, such as increased fertility, fecundity, and longevity in females. However, Gonçalves et al. (2024) emphasized the significant influence of male quality in this polyandrous behavior. Given the relevance of this pest in tomato agriculture, our study aims to elucidate the factors shaping these mating patterns, hypothesizing that engaging in multiple matings may considerably impact fundamental parameters, directly influencing the

reproductive performance of females. To address this question, we chose to perform matings with different males and the same male, aiming to investigate how variability in male quality may affect reproductive outcomes. Through the isolation of variables, such as the number of matings, mating status, age, and weight of individuals, we seek to comprehensively clarify these polyandry effects on the reproductive aspects of the tomato leafminer. Thus, this study aims to clarify the true influence of mating patterns on the reproductive process of *T. absoluta*, assessing oviposition, fertility, and longevity parameters in females of the species.

Materials and Methods

1. Plant growing conditions

The experiments and insect rearing took place at the Laboratory of Insect Behavior and Semiochemicals, Federal University of Viçosa (UFV), MG, Brazil. Tomato plants (*Solanum lycopersicum*, Vivaty variety) were employed for both oviposition and larval feeding of *T. absoluta*. Seeds were planted in plastic trays containing vegetable growing substrate (MecPlant R soil, MecPlant, Telêmaco Borba/PR-Brazil) and placed in a germination chamber (Tecnal TE-4000/1). Following this, seedlings with 2-3 leaves were transplanted into 3-liter pots with substrate conditioner and moved to a greenhouse maintained at a temperature of $25 \pm 2^\circ\text{C}$ and a relative humidity of $70 \pm 5\%$. Macronutrients were supplied every 7 days, with NPK fertilizer (10:10:10, Fertipar Group, Curitiba, PR-Brazil).

2. *Tuta absoluta* rearing

Tuta absoluta was reared under controlled environmental conditions in a breeding room, maintaining a temperature of $25 \pm 2^\circ\text{C}$, a photoperiod of L12:D12, and a relative humidity of $70 \pm 5\%$. Captured adults were housed in acrylic cages (30 x 30 x 30 cm) and provided with unrestricted access to a 10% sugar solution, along with tomato leaves for oviposition. Egg-laying plants were changed every two days and transferred to wooden cages (50 x 40 x 40 cm)

covered with organza fabric to facilitate proper air circulation. Upon hatching, larvae were nourished with tomato leaves stored in containers with water to retain their freshness. After the larval stage (lasting 15 to 20 days), pupae were carefully detached from the leaves and arranged in petri dishes (90 x 15 mm) within cages to support their development and eventual emergence as adult moths.

3. Sexing *T. absoluta*

The weight for both male and female *T. absoluta* was determined based on pupal weight, following the procedure outlined in Muller et al. (2016). Pupae, aged 2 to 3 days, were extracted from tomato leaves, sexed using the genital pore location method (Coelho & França, 1987), and weighed using a SHIMADZU AUW220D analytical balance. Weight ranges were established by weighing 200 pupae for each gender. The females used in the experiments had a pupal weight ranging from 2.10 to 3.43 mg. For the males, whose pupae had a mean weight between 1.89 and 2.66 mg, they were placed individually in 70 mL plastic cages sealed with organza mesh and observed until emergence.

4. Experiments

To elucidate the true influence of mating patterns on the reproductive process of *T. absoluta*, we assessed oviposition, fertility, and longevity parameters in females, with 25 repetitions for each experimental group. For this purpose, 75 females (25 for the experiment and 50 to change mating status) and 75 males were mated for Experiment A (polyandry: same male). For Experiment B (same male), 25 females and 25 males were used. Finally, for Experiment C (single mating), 25 females and 25 males were used, totaling 300 individuals across all experiments. The experiments will be detailed in the section below (see Table 1):

Experiment A: In this experiment, a single female was mated with different males. Initially, a virgin female was paired with a virgin male aged 1 day. Subsequently, the same female was mated with a different male aged 3 days, completing 1 mating event. Finally, the same female was mated with another different male aged 5 days, totaling 3 mating events.

Experiment B: In this experiment, females were mated with the same male. Starting with a virgin female and a virgin male aged 1 day, the process was then repeated with the same male at 3 days of age, resulting in 1 mating event, and later at 5 days of age, totaling 3 mating events.

Experiment C: In this experiment, females had a single mating event, starting with a virgin female and a virgin male aged 1 day.

To standardize the number and timing of matings, pairs were separated after each mating event and individually housed in 70 mL plastic containers containing crystal organza fabric for one day. To ensure copulation was recorded, we established a parameter of a minimum duration of 30 minutes, as this period is considered necessary for males to complete the spermatophore transfer process, according to Ouye et al. (1965) and Lee et al. (2014).

4.2. Oviposition, Fertility and Longevity

After mating, females were placed in 70 mL plastic containers sealed with crystal organza fabric containing two tomato leaves placed in a 1 ml glass vial with water for oviposition. Egg counting was conducted every two days concurrently with leaf replacement. The leaves were substituted until the death of the female. To analyze fertility, glass vials containing leaves and deposited eggs were kept in 2-liter plastic cages enclosed with organza nets, and newly hatched larvae were counted every two days until larvae ceased to emerge. Additionally, for longevity analysis, females were continuously observed from adult emergence until death, with daily recording. This approach facilitated understanding the effects of

polyandry while controlling variables such as weight, age, mating status, and the number of matings.

5. Statistical Analysis

Statistical analyses were performed using the R software (version 4.0.0; R Development Core Team, 2020). To assess the influence of multiple matings on *T. absoluta* females' fecundity (number of eggs per female) and fertility (number of hatched larvae per female) in each experiment (Table 1), generalized linear models (GLMs) with Poisson and binomial error distributions, respectively, were conducted. Subsequently, pairwise comparisons of Least Squares Means (emmeans) were performed to assess differences between treatments. For longevity analysis (days of female life), survival analysis with Weibull distribution was utilized.

Results

The impacts of mating with males of different mating patterns on egg laying, fertility, and female longevity were investigated, considering the number of copulations. (Fig. 4 A, B, and C). The results did not reveal significant differences in the number of eggs laid by females (Fig. A; GLMs with Quasipoisson family: $n = 25$, $F = 0.009$, $p > 0.05$) mated with different males (9.76 ± 0.95), the same male (9.89 ± 0.87), or a single mating (9.94 ± 0.96).

However, fertility was affected by polyandry (Figure 1A; GLMs with Quasibinomial family: $n = 25$, $F = 3.552$, $p = 0.02$). Females that mated with different males (0.706 ± 0.018) had a higher number of larvae from the eggs compared to females that mated with the same males (0.64 ± 0.01) or had a single mating (0.69 ± 0.01) (Figure 1B). Additionally, female longevity was not affected by multiple mating (survival analysis with Weibull distribution; $n = 25$, $\chi^2=497.23$, $p > 0.05$) (Figure 1C).

Regarding the oviposition rate of females mated with different males, no significant differences were identified among females mated with different males, the same males, or a single mating (survival analysis with Weibull distribution; $n = 25$, $\chi^2 = 2971.0$, $p > 0.05$). However, it was observed that in *T. absoluta*, the highest oviposition rate occurs in the first six days of female life (survival analysis with Weibull distribution; $n = 25$, $\chi^2 = 2493.2$, $p < 0.001$) (Figure 1D).

Discussion

Polyandry, a reproductive strategy, is often seen as providing direct and indirect benefits to females (Arnqvist & Nilsson, 2000). In our results, direct benefits of polyandry for female *T. absoluta* were not observed, similar to findings by Harano and Miyatake (2006) and Wang et al. (2005), who also did not observe significant differences in the number of eggs produced, hatched, and the longevity of females between multiple mating and single mating scenarios. However, it is worth noting that there are exceptions, as observed by Kawagoe et al. (2001) who noted a decrease in female longevity in the butterfly *Atrophaneura alcinous* (Klug, 1836) when mating multiple times. Previous research has also shown a positive relationship between female mating number and oviposition rate, fecundity, and longevity (Seth et al. 2002; Lee et al., 2004; Torres-Vila et al. 2013). It is important to note that the correlation between female mating and direct benefits, although observed in some studies, is not universal (Torres-Vila & Jennions, 2004). As emphasized by Torres-Vila & Jennions (2004), this relationship can vary significantly among different contexts and species, underscoring the importance of a careful approach in the methodologies used in studies on polyandry.

However, in contrast to our observations, Lee et al. (2014) highlighted benefits to females by allowing *T. absoluta* females to mate with virgin males instead of repeating mating with the same male. It is relevant to note that, while Lee et al.'s (2014) experiments involved

matings throughout the entire reproductive life of the female, our standardized approach consisted of only three matings, aiming to mitigate factors that could directly influence reproductive parameters, such as weight, age, mating status, and longevity. The standardization adopted in this study was crucial for controlling specific variables, as highlighted by Torres-Vila and Jennions (2004). By removing primarily the influence of age, weight, and mating status, studies indicate that the weight, delay, and quantity of matings by males can significantly affect the number of sperm transferred to females (Marcotte et al., 2007; Gonçalves et al., 2024). Furthermore, in insects, delayed mating can reduce the fecundity and fertility of females (Krann & Straten, 1988; Jones & Aihara-sasaki, 2001). Such alterations can result in changes in reproductive rates, such as fecundity, fertility, and longevity, or even decrease female receptivity (Lingren et al., 1988; Ringo, 1996; Rogers & Marti, 1996; Marcotte et al., 2007; Gonçalves et al., 2024).

However, considering that *T. absoluta* females receive more than enough sperm to fertilize their eggs in a single mating (Xu & Wang, 2009), our results suggest that polyandry in *T. absoluta* may be more related to the hypothesis of genetic benefits, such as increased offspring viability by promoting genetic variability, allowing the selection of favorable genes, mitigating the adverse effects of inbreeding, enhancing adaptive fitness, and facilitating responses to environmental changes (Arnqvist & Nilsson 2000; Jennions & Petrie, 2000; Gowaty, 2013; Parker & Birkhead, 2013). Jennions & Petrie (2000) emphasize that most rematings occur due to the identification of superior partners, resulting in genetic exchange.

Arnqvist & Nilsson (2000), in their review, along with other authors (Jennions & Petrie, 2000; Gowaty, 2013; Parker & Birkhead, 2013), discuss the possible undervaluation or underestimation of the direct effects of multiple matings on females and highlight the importance of considering various crucial aspects for reproductive success, such as genetic variability, sperm competition, mate choice, and other factors that play significant roles in

female reproductive dynamics. Harano et al. (2006), as well as Thornhill and Alcock (1983), trace emphasis to the idea that females may be coerced into accepting superfluous matings by persistent males, reducing the costs of male harassment (Clutton-Brock and Parker, 1995; Arnqvist, 1997). This dynamic is explained by the convenience polyandry hypothesis, suggesting that females mate to reduce the costs associated with sexual harassment (Thornhill & Alcock, 1983). Thus, our studies reinforce the idea of Jennions & Petrie (2000) that the isolated analysis of reproductive parameters, such as oviposition, fecundity, and longevity, may not be sufficient to fully unravel the impacts of multiple matings. Delving into the investigation of interactions between variables such as sperm competition and genetic diversity can provide a more comprehensive understanding of the reasons underlying polyandry in *T. absoluta*.

Through the experiments conducted by Lee et al. (2014), it was observed that oviposition peaked in the early days of female life, with the highest proportion of eggs being deposited in the first 7 days. However, in our experiments, no differences were observed in the oviposition rate between females that mated only once and those subjected to multiple matings. Similarly, the oviposition patterns in our experiments resemble those found by Lee et al. (2014) in *T. absoluta*, with females ovipositing until approximately 25 days of age, as also observed by Imenes et al. (1990) and Uchôa-Fernandes et al. (1995).

Our results did not reveal a direct influence of mating patterns on these crucial reproductive parameters in *T. absoluta*, contrary to some studies (Seth et al., 2002; Lee et al., 2004; Torres-Vila et al., 2013). Therefore, it is crucial to explore more deeply the effects of these matings, considering factors such as the standardization of variables and the possibility of other uncontrolled elements playing a relevant role. Since multiple matings can also disadvantage females by increasing exposure to pathogens, directly leading to a decrease in their survival (Arnqvist and Nilsson 2000; Gowaty, 2013; Parker & Birkhead, 2013). A more in-depth understanding of this apparent discrepancy requires not only a careful analysis of

standardized variables but also the consideration of uncontrolled elements that may play a significant role in these reproductive dynamics.

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Table 1: Mating patterns of *T. absoluta* individuals for each experiment.

Experiment (Matings)	Group	Female Mating Status	Female Age	Male Age	Male Mating Status
A1	Polyandry/Different Males	Virgin	2 days	1 day	0 matings
A2	Polyandry/Different Males	1 mating	4 days	3 days	2 matings
A3	Polyandry/Different Males	2 matings	6 days	5 days	3 matings
B1	Same Male	Virgin	1 day	1 day	0 matings
B2	Same Male	1 mating	4 days	3 days	2 matings
B3	Same Male	2 matings	6 days	5 days	3 matings
C1	Single Mating	Virgin	2 days	1 day	0 matings

* The term "matings" refers to the number of mating events. "Virgin" denotes a female or male that has not mated. The age indicates the days since emergence. The male mating status specifies the number of previous matings.

Figure

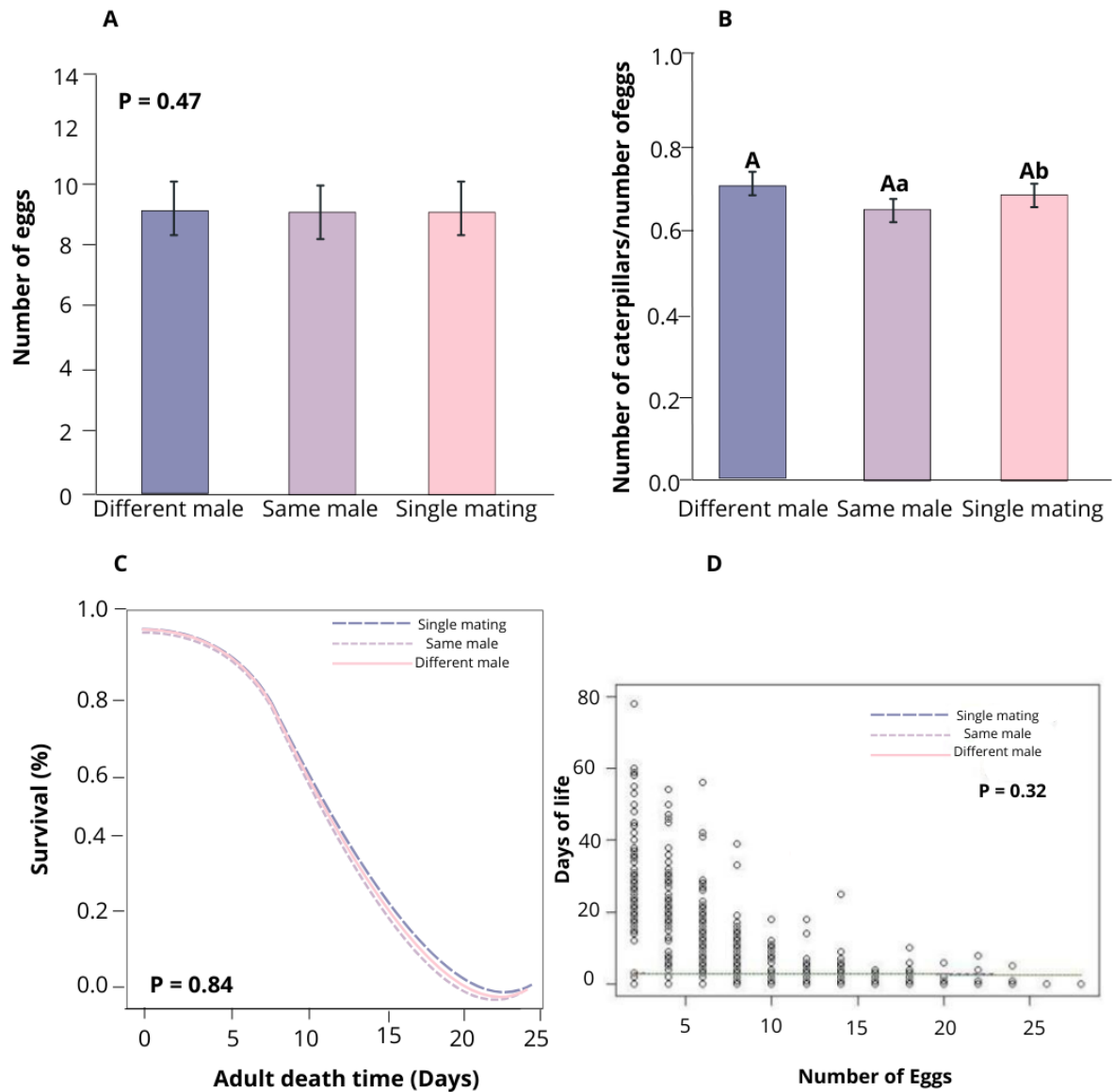


Figure 1: Reproductive Characteristics of *T. absoluta* Females Mated with Different Males (Polyandry), Same Males (Same Male), and Single Mating. A= Fecundity (Number of eggs); B= Fertility (Number of larvae/number of eggs); * P values < 0.05 indicate statistical differences (GLM; Quasipoisson and Quasibinomial family); C= Longevity; D= Oviposition Rate. * P values < 0.05 indicate statistical differences (Survival analysis and Weibull distribution).