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Bioassay

Mating status affects females feeding behavior but does not the encapsulation response in the field cricket, *Gryllus* (*Gryllus*) *assimilis* (Fabricius, 1775)

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Abstract. Mating can trigger different effects on the physiology and behavior of animals, especially in females. These effects can be either beneficial, such as boosting the immune response, or harmful, such as decreasing survival, for example. Cricket females are among the most used models for understanding these life history trade-offs. We recently demonstrated that mated females have a slight cost of reproduction in survival that could be explained if they differed in resource consumption compared to unmated, being able to avoid putative damages of reproduction on self-maintenance. To test whether mating status modulates feeding behavior and self-maintenance (i.e., immunity), we performed two experimental blocks: In the first, we measure the food intake of both virgin and mated females of *Gryllus* (*Gryllus*) *assimilis* (Fabricius, 1775). In the second, we compared the ability to mount an acute immune response (encapsulation) against a nylon filament implant, a widely used method to challenge the insect immune system. Results showed that mated females did not increase food consumption compared to pre-mating period, however, the virgin group (control) drastically decreased the amount of food ingested. Furthermore, no difference in immune response was found between mating status. As mated females ingested more food than those virgins of the same age, we suggest the feeding behavior (i.e., resource acquisition) as one of the used behavioral strategies to maintain immune ability, high oviposition rates, and survival of mated females of *G.* (*G.*) *assimilis* when fed *ad libitum*.

Keywords: Immunity; Trade-off; Reproduction; Food consumption; Fitness.

The effects of mating play a key role in the evolution and plasticity of physiological traits of organisms, including the trade-offs between reproduction and self-maintenance, a tenet of life history evolution (Stearns 1989; 1992). These mating effects are either beneficial or harmful to females and it is well established that there are complex intra- and interspecific aspects that interact to display this automated physiological “mating response” (Flatt 2011; Schwenke et al. 2016; Kelly 2018).

Insect females, particularly crickets, are a very useful model to understand the dynamics of reproductive trade-offs and have been contributing to this purpose for decades (Zera et al. 1998; Shoemaker & Adamo 2007; Park & Stanley 2015; Miyashita et al. 2020; Limberger et al. 2021; 2022; Treidel et al. 2021). Resource allocation and acquisition are processes that underlie trade-off dynamics that are closely related to environmental profile but not restricted to that. Some studies report that trade-offs related to reproduction (e.g., cost of reproduction) are masked or undetectable when resources are plentiful (Moret & Schmid-Hempel 2000; Shoemaker & Adamo 2007). Mated females could readily increase food consumption to support fertility and self-maintenance (e.g., immunity), however, there is a gap in the experiments aimed to empirically support this hypothesis in this important insect model, by comparing the food intake of virgin and mated crickets.

We have previously demonstrated that females of Jamaican field crickets, *Gryllus* (*Gryllus*) *assimilis* (Fabricius, 1775), showed a modest cost of reproduction perceived in survival of mated females (about a week in the maximal lifespan), although the oviposition rate of these mated females was approximately four times higher than that observed in virgins of the same age when experimentally kept under *ad libitum* diet (Limberger et al. 2021). Mating has an immediate effect on the reproductive system of field cricket females, increasing the oviposition rate as a result of male-derived substances, as prostaglandin, that are transferred during copulation (Worthington & Kelly 2016). As mating obligatory triggers oviposition at high rates, our previous results regarding the cost of reproduction in survival could be explained if mated females consumed more food than virgins, sustaining reproductive output and self-maintenance simultaneously from an energetic point of view. However, we did not empirically validate this hypothesis, which could clarify our understanding of resource acquisition and the allocation among expansive traits. A related study using females of Mediterranean field cricket, *Gryllus* (*Gryllus*) *bimaculatus* De Geer, 1773, showed that the period following mating is characterized by changes in dietary preference (i.e., food with higher protein content was preferred) (Tsukamoto et al. 2014). In this way, information on feeding behavior of virgin and mated females of *G.* (*G.*) *assimilis* can be very useful to enable comparisons across species and life history strategies linked with the reproductive process in female insects.

Different effects of mating on immune response in cricket females have been also reported. For instance, in the ground cricket *Allonemobius socius* (Scudder, 1877), mating reduced the number of circulating hemocytes, bacterial lytic ability, and the encapsulation response (Fedorka et al. 2004). It is important to keep in mind that the encapsulation response against a foreign material (e.g., nylon monofilament) can be interpreted as immune defense strength, as it includes activation of both cellular and humoral components of the immune system (Siva-Jothy et al. 2005). For this reason, this method is widely employed in studies regarding the costs of life history traits in insects by simulating the invasion of a novel intruder (Schmid-Hempel 2005; Limberger et al. 2022). The degree of immune response can be easily quantified by calculating the melanin covering the implant in photographs, which darkness of the implants reflects the efficiency of the immune response (Fedorka et al. 2004). Negative effects of copula were also noted on the encapsulation ability in the house cricket *Acheta domesticus* (Linnaeus, 1758) (Bascuñán-García et al. 2010). In contrast, mating enhanced resistance to bacterial infection in the field cricket *Gryllus* (*Gryllus*) *texensis* Cade & Otte, 2000 (Shoemaker et al. 2006; Worthington & Kelly 2016), however, the number of matings had no apparent effect on immunity in another field cricket species, the *Gryllus* (*Gryllus*) *vocalis* Scudder, 1901 (Gershman 2008). So, it is evident that experimental studies using different immune elicitors as well as other cricket species are needed to enable suitable comparisons on this topic, which would be very helpful in the understanding of life history evolution and diversification of physiological strategies to deal with immune assaults. The putative effects of mating on immune responses and feeding behavior are completely unknown for *G.* (*G.*) *assimilis*. Moreover, most studies about nutritional characteristics of trade-offs were conducted with polymorphic-winged field cricket species (e.g.: Clark et al. 2015; Stahlschmidt & Adamo 2015; Treidel et al. 2021), in which long and short wings morphotypes can naturally occur, however, in *G.* (*G.*) *assimilis* only long-winged morphotype are found. This biological difference among field cricket species interferes with how organism allocate their energy to dispersal, reproduction, and self-maintenance, for instance.

To test whether mating status affects feeding behavior or the immune response, two independent experiments were conducted. In both experiments, females were sampled from a long-term cricket colony housed in the Laboratory of Terrestrial Invertebrates at the Institute of Biological Sciences in Universidade Federal do Rio Grande (FURG), Brazil. Crickets were maintained under standard controlled conditions of room temperature (27 ± 1°C), 12 h light:12 h dark cycle, and humidity of 70% ± 10% (Limberger et al. 2021). Experimental females were 15 ± 3 days old (adult) when they were randomly allocated to establish a mated or virgin group (with no differences in size or mass). After the copulation period (mated had spermatophore attached) all females were weighed, then were individually housed in plastic pots (12.5 cm × 9.5 cm) for experimentation. All experimental procedures were done under standard controlled conditions in the rearing room, with food and water provided *ad libitum*. Wet cotton balls were used for water consumption and as standard oviposition substrate (Limberger et al. 2021; 2022).

To determine food consumption, we followed the protocols from our previous work (Limberger et al. 2022), in which a known weight of standard dry ground cat food (high protein content: 36.5%) (GoldeN, PremieRPet®, São Paulo, Brazil) was daily replaced for each female (virgins, n = 15; mated, n = 14). After 24 allowing consumption, the uneaten food was identified and stored in an oven for the next 24h to obtain a dry weight. The difference between the dry weight of uneaten food and the dry weight of given food was determined as food consumption and was expressed as mass-specific consumption (g dry food/g cricket live mass/day). As a control, we measured the food consumption of both groups for three days before the experimental changes in mating status, which gave us an average consumption per female per day (i.e., pre-mating), ensuring that any change in feeding behavior observed after mating would be a result of different mating status and not due to experimental bias (e.g., individual mass) (Fig. 1a). Mated females were allowed to mate *ad libitum* for 24h in a large plastic container (65 × 44 × 40 cm) with virgin males (2:1) of the same age. After this period, food consumption was verified for three consecutive days in both mated and virgin groups (Fig. 1a), because it includes the period of most conspicuous oviposition rate in mated females of this species (Limberger et al. 2021), which suggests a high resource demand to oogenesis and egg-laying behavior. Comparisons across consumption by mated and virgin females were statistically analyzed by *t*-test, Mann-Whitney, and Wilcoxon tests depending on the distribution of data.

Results of feeding behavior showed that there was no difference in food consumption between groups during the pre-mating period when all experimental animals were indeed virgins (Mann-Whitney, p = 0.35) (Fig. 1b). After the mating period (mated group), the food consumption was statistically different, in which mated females ate more than virgins of the same age (*t*-test, p < 0.01) (Fig. 1c). At this point, mated females continued eating a similar amount of food to when they were virgins (Wilcoxon test, n = 14, p = 0.10), however, the virgin group showed a reduction in food intake when compared to their previous assessment (Wilcoxon test, n = 15, p < 0.001).

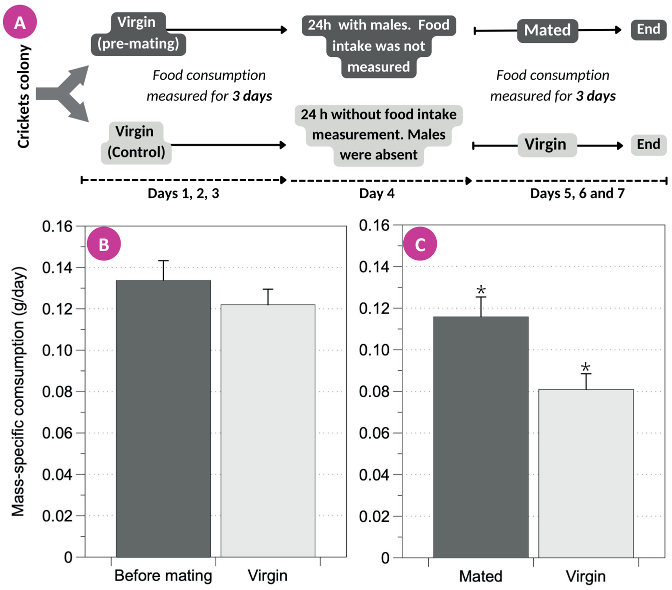

Figure 1. Experimental design and mass-specific consumption of standard food (g dry food/g cricket live mass/day) by females of *Gryllus* (*Gryllus*) *assimilis*. Experimental design was chronologically expressed; Days are referred to experimental period, not age (dashed arrow) (a). Comparison between food intake of experimental groups: virgins and mated before copulation (3 days), when all females had the same mating status - Control (b). Food consumption of mated and virgin females after copulation period for those mated (3 days) (c). Asterisks denote the statistical difference between the mating status calculated by t-test (p > 0.01).

To compare the immune response of virgin (n = 20) and mated (n = 20) females against a nylon monofilament, new mated and virgin treatments were reestablished and maintained under standard conditions of experimentation as described earlier. In this experiment, mated females were allowed to mate *ad libitum* for 72h in a large plastic container (65 × 44 × 40 cm) with virgin males (2:1) of the same age, following immunological experiments of Limberger et al. (2022) using this species**.** Specimens were cold anesthetized on ice for circa 15 min, then we inserted an aseptic implant of nylon (5-mm long × 0.3-mm diameter) using a sterile pin to make a wound on the left ventral side of each cricket, in the pleural membrane between the second and third sternites where half (2.5-mm) of filament was left outside the body to be removed later (Bascuñán-García et al. 2010; Limberger et al. 2022). Before surgery, all surfaces, surgical instruments, and the cricket's cuticle were properly sterilized with ethanol for asepsis. Following Bascuñán-García et al. (2010) and a pilot experiment with *G.* (*G.*) *assimilis* females, we removed the implants from both virgins and mated females 24h after filament insertion, because at this time there was a complete encapsulation response in the inserted portion. Implants were then individually identified and stored in 70% ethanol for further analysis. The darkness of implants was determined according to Rantala & Roff (2005), in which digital photographs were taken from three different angles of each implant under a stereomicroscope (Leica EZ4, Leica Microsystems, Wetzlar, Germany) coupled with a camera. Encapsulation rate was analyzed as the mean gray value of reflecting light from the three images per implant per female (average), using ImageJ software, so a darker value means a greater immune response. The darkness value was obtained by subtracting the value of a control filament (not inserted) from the darkness of the implanted filaments (Rantala & Roff 2005). A comparison of immune response between mated and virgins was done by *t*-test.

The immunological experiment revealed no statistical difference regarding immune response against a nylon monofilament implant between mated and virgin females of *G.* (*G.*) *assimilis* 24 h after surgical implantation (Fig. 2a, b). Encapsulation response seemed to be very effective (Fig. 2a), however, no effect of mating was perceived on this immunological trait (Fig. 2b).

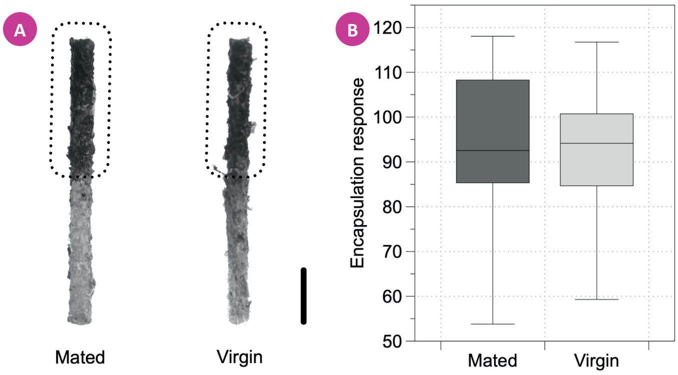

Figure 2. Immune (encapsulation) response against a monofilament implant from mated (n = 20) and virgin (n = 20) females of *Gryllus* (*Gryllus*) *assimilis*. Nylon filament and encapsulation: dotted area is referred to the inserted portion. Scale bar: 1 mm (a). Boxplot (median, interquartile range, higher and lower observations) of encapsulation response and respective mating status (b). Statistical comparison was done by Student's t-test.

Results regarding feeding behavior support our hypothesis about increased resource demand as a side effect of first mating in females of *G.* (*G.*) *assimilis*. Virgin females do not contribute to population fitness in the wild, but they are very useful as an experimental tool in studies that investigate trade-offs and costs of reproduction as shown here. Interestingly, we did not notice an increase in food consumption by mated females, however a clear decline in feeding was observed in virgin crickets of the same age, which strongly suggests that most of the consumed resources are allocated to the eggs and oviposition behavior in mated females, as recently pointed out in a study regarding the costs of a chronic immune response and food intake in these female crickets (Limberger et al. 2022). A hypothetical explanation for the lower food consumption by virgins, in addition to the logical reasoning of lower energy expenditure in egg production and laying, comes from experiments with *A. domesticus* and *G.* (*G.*) *bimaculatus* crickets (Clifford & Woodring 1986; Woodring & Lorenz 2007). It was suggested that the digestive tract of virgin females face a space constraint in the abdominal cavity since their ovaries are naturally increasing in size over time, and the oviposition rate is substantially lower than that of mated females. So, this might be one of the reasons why virgin females lay some unfertilized eggs regularly as seen in Limberger et al. (2021).

Furthermore, both mated and virgin females were reared in a nutritionally rich-environment (*ad libitum*), which could allow them to store resources in the fat body, where most immune-related proteins are synthesized (Skowronek et al. 2021). A resource-limited condition since early age would be helpful in this experimental context, as a nylon implant leads to a reduction in food intake in females of *G.* (*G.*) *assimilis* reared with food *ad libitum* (Limberger et al. 2022).

Unlike house crickets (Bascuñán-García et al. 2010), we found no effect of mating on encapsulation using the same methodology to challenge the immune system of females, which points to differences in how these cricket females allocate their energy to reproduction and self-maintenance (e.g., immunity). Moreover, it is important to note that a stronger immune response may not confer greater fitness, as the resources used by this trait would divert resources that could be allocated to reproduction (Drayton & Jennions 2011; Limberger et al. 2022). The higher food consumption by mated insects helps to explains the very low costs of reproduction perceived on survival of females of this species (Limberger et al. 2021). These findings further expand our understanding of the characteristics of the post-mating period on behavioral and immunological traits of female insects. We reinforce the use of multiple analyses such as behavior (e.g., feeding, locomotion, laying) and different mating statuses (e.g., virgin, early and late reproduction) to clarify the dynamics of life history events among female crickets.

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Authors' Contributions

GML, LEMN, and DBF designed this experimental research. GML conducted all the experiments and analyzed the data with contributions of LEMN and DBF. GML wrote all the manuscript versions. The final manuscript was revised and approved by all authors.

Conflict of Interest Statement

We have no conflict of interest to declare.

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