Merge

Volume 2 Article 7

2018

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The W Mississippi University for Women
Founded 1884 Coeducational since 1982

Volume 2
Spring 2018

Merge:
The W's Undergraduate Research Journal

Volume 2
Spring, 2018

Compromised Mating Responsive Gene CG18125 Increases Fertility in *Drosophila Melanogaster*

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Abstract

Sexual conflict is understood as differential optimal reproductive fitness between the sexes of a species, which can lead to an evolutionary arms race between the sexes. Typically,males evolve mechanisms to induce females to mate more often than is optimal, and females are selected to inhibit the effects of male manipulation. This antagonism, known as sexual conflict, has shaped the interactions of virtually all sexual species. Understanding the genetic basis of these conflict-based characteristics is key not only to increasing our understanding of sexual conflict but also to our understanding of the reproductive physiology and behavior of species. *Drosophila melanogaster* males and females possess a gene that codes for a putative serine endopeptidase, CG18125, which may mediate male sexual conflict mechanisms through influencing female fecundity and fertility. We hypothesized that heightened expression of CG18125 post-mating was due to its defensive purpose toward male manipulation, and that by deactivating the gene's functionality, we would increase fecundity and fertility as compared to that of normal female serine endopeptidase activity, thereby demonstrating that the gene in females acts as a mediator for male manipulation. Alternatively, we hypothesized that the drastically increased post-mating expression of the gene product may in fact be triggered by males as a way of ensuring an increased level of egg production, meaning that when the gene is absent, female egg production might decrease. In order to acquire this data, we performed a targeted suppression of expression within the female reproductive tract by RNA interference

(RNAi), which inhibited serine endopeptidase functionality, and recorded day-to-day egg deposition as well as viability counts for 12 days. Results demonstrated that there was no significant difference for or against fecundity but that experimental disruption of serine endopeptidase activity led to increased fecundity. However, experimental females did show a significant rise in egg production when compared to control females, on the first few days of egg laying. These results show that CG18125 expression does not affect fecundity numbers, but instead that it downregulates fertility. It is possible that CG18125 plays a role in physiological female protection by allotting a balance of egg production, and/or that males prosper from a shorter offspring generation time when the gene does not function correctly.

Introduction

Males of many species have been shown to optimize their own reproductive rate by manipulating female reproductive rate by any evolutionary means that arises. Because these male manipulations sometimes benefit males at a cost to females, it follows that females should, in turn, evolve means to interfere with or inhibit male tactics of this type, regardless of the form they take [1]. Proteases have a key role in sexual reproduction across a broad range of taxa, in some cases modulating key post-mating molecular interactions and, ultimately, critical reproductive physiological processes [2]. This is consistent with the hypothesis that females respond in their own interest even when males insert enzymes or proteins during mating that might otherwise cause females harm; some specific examples are reviewed in Mack and Bender [3], such as increased ovulation, mating inhibition (also known as a refractory period) after initial mating, and varying consequences for sperm use and storage. Heightened evolutionary change has been observed in these proteins, and is thought to be a consequence of sperm competition as

well as sexual conflict [4]. Mack and Bender [3] sought to identify possible genes expressed within the female reproductive tract that either up or down-regulated post-mating and potentially indicated sexual conflict mediation, which would in turn support the existence of genes with this purpose. Our research here focused on the gene, CG18125, which has been found to be dramatically up-regulated immediately following mating [3, 5], and its serine endopeptidase protein product. Specifically, we wanted to test for a possible role for this serine endopeptidase in female defensive adaptations.

A population genetic survey of proteases, protease inhibitors, and proteolysis expression (n = 44 individuals over 2 species, *D. melanogaster* and *D. simulans*) established that sexual selection did in fact play a key role in the evolution of the experimentally tested molecules [6]. This suggests that sexual conflict is ongoing in response to male mate competition by means of sexual selection by females. Antagonism at the molecular level between males and females is one clear manifestation of this process. This raises the question as to where this sexual conflict stems genetically. Lawniczak and Begun [5] utilized quantitative trait locus (QTL) in order to plot areas of *Drosophila* genome that play a role in female tendency to use first male sperm rather than second male sperm, female refractoriness mating after an initial mating, and adolescent fertility. Their data suggest that specific gene locations are triggered for the expression of biomolecules that contribute to this molecular antagonism. We targeted genes that are involved in the potential expression of proteins that induce female defense responses to this male-induced sex conflict.

Sexual conflict occurring within the females must then be targeting particular physiological regions within the female's reproductive tract. From this, one expectation is that specific structures within the female reproductive tract of *D. melanogaster* play key roles in the

outcome of sexual reproduction and are therefore likely male molecular targets. One likely possibility is that females control the fate of sperm, whether it be fertilization or potentially disregard in preference of another's sperm [7], a tactic not limited to the genus, *Drosophila*, and, no doubt, largely targeting female sperm storage and fertilization structures, especially the spermathecae, regardless of taxa[8]. Consequently, the spermathecae are a likely area of molecular sexual conflict, a region that the male might try to control in order to have a better chance at successful progeny output.

Here we focus on spermathecal regions that regulate transcription of two genes in spermathecal secretory cells (SSC) [9]. SSC are necessary for bringing sperm to the spermathecae but not for keeping sperm there [9]. SSC ablation lowers sperm motility in the seminal receptacle and creates ovoviviparity, the tendency to withhold fertilized eggs and even allow them to grow within the female [9]. Those authors proposed that endopeptidases prevent the transport of sperm to the site of fertilization, thus inhibiting internal fertilization and live birth which can lead to severe female health compromise. In fact, without these dual sperm storage and maintenance cells, females will give live birth instead of dropping eggs as they might under natural circumstances [10]. These effects are more important to females, since females must deal with the effects on their reproductive physiology as a consequence of mating. By contrast, males bear no such cost as they simply achieve increased reproductive success. Similarly, mating stimulates physiological and behavioral female modifications by transitioning virgin females from non-offspring producing to offspring producing mated females, likely as a means of increasing fecundity assurance [11].

Sexual conflict can be assessed through RNAi, which provides the experimental basis for determining the effects of disrupting the functionality of certain genes and therefore their gene

products. RNAi was used to remove the functionality of seminal fluid proteins (SFPs) CG10586, and showed that impacted ovulation as well as sperm retention, and that its lack of functionality provoked a decrease in egg count, a rise in female mating rate, and strange sperm utilization intervals, demonstrating the protein's role in upkeep of sex peptide [12]. The research indicates not only that sexual conflict can be observed in a lab setting where specific genes of interest are targeted, but that RNAi can be used as the mechanism in assuring that only one gene in one region is being affected, a mechanism used in our research.

We hypothesized that the disruption of the functionality of serine endopeptidases, our hypothesized female defensive mechanism in mating, would increase the level of fecundity and correspondingly fertility in female fruit flies. Our use of genetically modified *Drosophila* lines was chosen with the exclusive purpose of inhibiting expression of our candidate gene of interest, CG18125, by specifically targeting it. Since RNA is the intermediate between the DNA sequence and the enzyme product, the fly lines that we used would inherently interfere with the RNA step in just the reproductive tissue of interest, meaning that its inhibition would not affect any other region of the female. RNAi lines are fly lines where a particular sequence of DNA has been inserted in proximity to the gene of interest. The result of this manipulation is that during translation of the sequence, and through stimulation by the GAL4 driver line, a complementary strand of RNA is assembled. This RNA will readily bind to any mRNA transcripts produced as a result of the candidate gene in order to produce double-stranded RNA. Since double-stranded RNA trigger cellular mechanisms to degrade them, they serve as a genetic regulatory tool. The mRNA that have been targeted will consequently be torn apart by the cellular regulatory machinery resulting in the targeted gene's loss of functionality and therefore the loss of the protein of interest [13].

By using this RNAi tactic, we found that gene CG18125 did not in fact affect the fecundity number, but that experimental females did experience an extreme peak in egg production on the first few days of egg laying, in contrast to the relatively constant egg production seen in the control group of females. More interestingly, however, was the fact that fertility was significantly increased in the experimental population on three days and significantly decreased on three days. These results indicate that the hypothesis was rejected in terms of fecundity data, but that fertility was in fact affected by a seesaw pattern of production height between control and experimental females, potentially due to physiological restrictions.

It is important that sexual conflict, its cause, its purpose, and its effect in various species be understood not only for the furthering of the scientific field, but also for the furthering of species based physiological knowledge. Our data may serve as a widening basis for comprehension of the genetic grounds for post-mating reproductive physiology in females across a broad range of taxa with genetic similarities to *Drosophila melanogaster*. This research has furthered previous and ongoing research with regards to female and male sexual conflict. Our research was able to clarify and categorize gene CG18125 and its role as a female defensive gene, yet there is still more to learn about its overall purpose. This study may fuel further research into female and male sexual conflict among other species. It may lead to similar correlations being drawn of sexual conflict in humans and other taxa with similar genetic compatibility as *Drosophila melanogaster*, possibly impacting human understanding of fertility and specifically the variables that relate to aspects of species-wide production and successful viability of offspring.

Results

Control females oviposited at a relatively steady rate (Figure 1) but less so on days 1, 11, and 12 ($p < 0.00406$ in each case, as verified by the Bonferroni Dunn-Šidák test method, where $n = 37$).

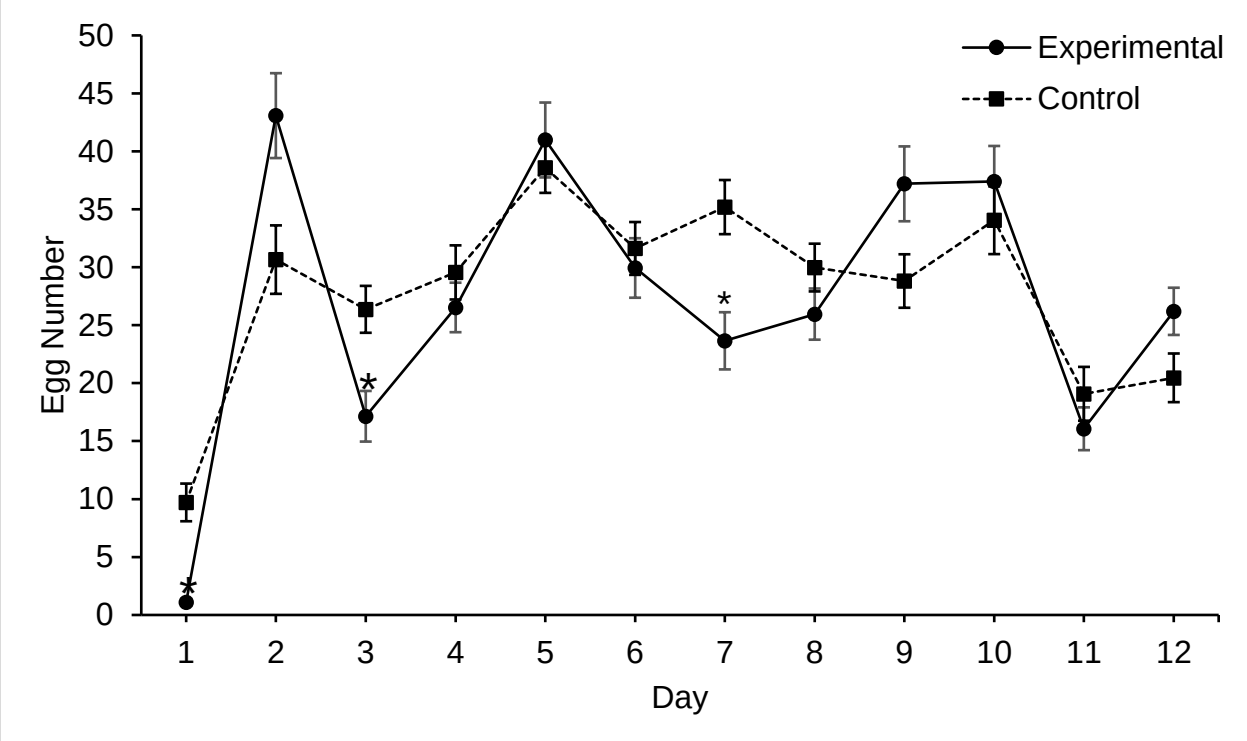

Figure 1. Control and experimental combined fecundity daily means, where experimental females had gene CG18125 obstructed by RNAi and control females CG18125 functionality remained intact. As verified by the Bonferroni Dunn-Šidák test method value of 0.00406, days 1, 3, and 7 showed significant differences between control and experimental egg output (control, $n = 37$; experimental, $n = 45$). *signifies $p < 0.00406$

Experimental females oviposited at a similar rate to control females (daily mean = 336.9556, standard deviation = 72.893, standard error of the mean = 11.98353, $n = 45$; $p = 1.0$), with some significantly lower production on days 1, 3, and 7.

Experimental female fertility showed significantly lower rates on days 1, 3, and 7 (Figure 2), but significantly higher rates on days 2, 9, and 12 ($p < 0.01$ in each case, as verified by the Z-test and corrected by the Bonferroni Dunn-Šidák test method, where $n = 45$).

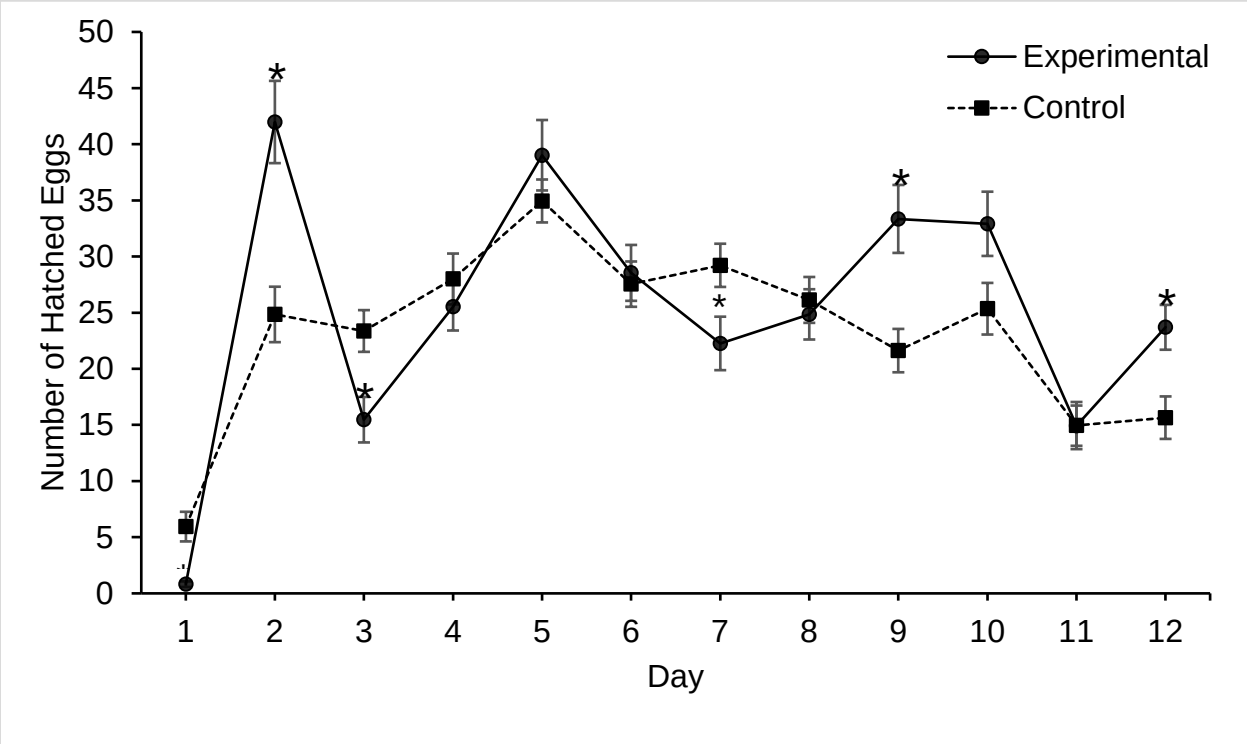

Figure 2. Control and experimental combined fertility daily means, where experimental females had their CG18125 gene functionality obstructed and control females CG18125 functionality remained intact. Z-test, corrected by Bonferroni Dunn-Šidák test method value,revealed days 1, 2,3,7,9, and 12 demonstrated significant differences between control and experimental fertility.(control, $n = 37$; experimental, $n = 45$)* signifies $p < 0.01$

Experimental females demonstrated variation from the control females in six of the twelve days (daily mean = 315.0222, standard deviation = 97.34463, standard error of the mean = 14.51128, $n = 45$; $p = 1.0$) with some significantly lower rates on days 1, 3, and 7 as well as significantly higher rates on days 2, 9, and 12.

Discussion

No difference existed between the two treatments for total fecundity; though fecundity data revealed that days 1, 3, and 7 were significantly greater for control than experimental females. Fertility data revealed that days 1, 2, 3, 7, 9, and 12 were significantly greater for either control or experimental females. Fecundity was greater in control than experimental femalesin each cased (days 1, 3, and 7). We found fertility to be more variable among treatments where days 1, 3, and 7 were significantly greater for control females and days 2, 9, and 12 were significantly greater for experimental females. These results may be due to the effects of a role for CG18125 in sexual conflict, but as of now, the data seems to demonstrate a sees a relationship in experimental fertility where high output at one time consequentially results in lower output on subsequent days and vice versa.

Our data indicate a potential role for CG18125 in sexual conflict, but suggest a potentially more complicated story. It is possible that the gene still acts as a defensive mechanism for females in preventing early onset of a huge production of eggs, as seen by the Day 2 of the experimental females, leading to possible physiological harm to females with the defective gene. However, these results suggest that fecundity was unaffected by the gene's downregulation. In fact, egg production was higher for control females, an observation that initially seems counter-intuitive given our conflict hypothesis. Further experimentation will be required to resolve this apparent contradiction. Our results may suggest an advantage for males who mate with females that contain the defective gene. This advantage is due to this early, explosive production of eggs, meaning that the generation time for subsequent progeny will be shortened. On significant days of fecundity, the lower egg production by experimental females may be explained by the previous days where extremely high egg output led to a crash on days

thereafter. This reasoning stands for fertility as well, where an almost seesaw-like pattern is established. When massive experimental female egg output occurred, we saw substantially lower egg production in comparison to the control females. This may be due to a physiological restriction of the female to lay a rational number of eggs within a given time. Likewise, on the days of high output, these eggs are deposited so early on post-mating, that males are able to take advantage of younger, healthier, and potentially higher quality eggs for their future offspring.

Given our results, future work should focus on fertility over fecundity and delve into the reasoning behind this effect. We note that our fecundity results are not comparable to data from Schnakenberg et al (2012); where they did not assess fertility in relation to fecundity [7]. Future experiments should be designed to test for advantages and disadvantages for both males and females in response to higher fertility rates with early onsets of massive egg production in contrast to the same fecundity rates with steady egg production over the same period of time and lower fertility, including dissection and both physiological and molecular analysis of the spermathecal glands to unequivocally demonstrate that CG18125 was definitively inhibited in those tissues.

Methods and Materials

Drosophila Stocks and Fly Husbandry

RNAi, genetically constructed *Drosophila* lines, specifically line 15762, were obtained from a lab in Vienna, Austria. Control line 60,000 was similarly acquired from the Vienna lab. The Send 2- Gal 4 driver line was received from Dr. Mark Siegal's lab at New York University.

In production of non-functional CG18125 gene expression, virgin females, no older than 3-5 days, from line 15,762 were crossed with males of the Send 2-Gal 4 driver line for several

days. This cross confirmed non-functional gene expression in the spermathecal endothelium at the correct time and place of *Drosophila* development, and warranted progeny acquisition of both the Gal-4 driver as well as the UAS-RNAi, thereby establishing the experimental RNAi population.

Due to the stability of RNAi lines, their consequent upkeep was identical to that of any normal fly stock. These flies along with the 60,000 line and other fly stocks were kept at a constant incubation temperature of 23˚C and approximately 80% humidity along with a 12 hour light/dark cycle. All fly stocks were maintained in standard vials and bottles with ample fresh food.

For the experiment, collections of the experimental females were completed no more than a few hours after emerging, the females were isolated 5 per vial for 4 days, and upon confirming their virgin status, they were mated in a vial with 2 standard line 60,000 males of the same age and population for two days. The control line 60,000 females were treated in the same manner and allowed to mate in the same fashion. All vials contained ample food, this time consisting of a grape juice agar media on a small tasting spoon, for both experimental and control mated groups and their subsequent offspring. Males of both the experimental and control groups were removed after two days, while fresh tasting spoons were removed and counted every day for 12 days for fecundity and every other day for fertility. For the first 2 days, females and males were transferred together to fresh vials and media, and upon day 3, males were extracted separately and females were isolated to fresh vials and media every day for the remaining 10 days.

Media Preparation

Experimental and control group media consisted of the combination of microwaved 75 ml of water with 2 g of agar and the combination of 25 ml of boiled dark grape juice concentrate

with 0.6 g of sugar. These two mixtures were combined while still hot and poured into small tasting spoons. Once the media on each spoon cooled, one unit of yeast was applied to each spoon and subsequently placed in a freshly cleaned vial for either an experimental or control female to be placed in.

Fecundity and Fertility Microscopy

Every day for the 12 day experimental process, eggs were counted under a standard dissecting microscope at 2 PM and subsequently transferred by pipet to a fresh vial. This data was used for the fecundity outcome. Fertility was measured every other day for each day eggs were laid, in order to observe the number of viable offspring hatched under the dissecting microscope.

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