Characterization of courtship behavior and copulation rate in \textit{adp}^{60} mutant \textit{Drosophila melanogaster} (Insecta: Diptera: Drosophilidae)

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Abstract

Many metabolic consequences of the excess lipid storage observed in obesity have been documented in humans and mammals, but the effects of an obese phenotype on reproductive behavior are less understood. \textit{Drosophila melanogaster} (Meigan, 1830) is well suited to study the ramifications of excess energy storage as they are commonly used to explore the genes important for energy utilization. However, little is known about the reproductive behavior of mutants in these metabolic genes. Therefore, we tested, for the first time, courtship of the naturally occurring adipose (adp\textsuperscript{60}) mutation which accumulates excess triglycerides and glycogen. Adipose mutants showed decreased courtship behaviors as well as copulation rate. The decrease in copulation rate could in part be explained by the decrease in courtship behaviors such as wing scissoring and wing vibration. Taken together, the data presented here suggest that the \textit{adp}^{60} mutants may negatively affect male fly reproductive behavior and provide a system to further our understanding of the genetic and metabolic control of reproductive behaviors and reproductive potential.

Keywords: Adipose mutant, adp\textsuperscript{60}, courtship, lipid, glycogen

Introduction

Obesity is a condition whereby excess fat is stored in the body and affects almost 40\% of the United States population in 2015–2016 (CDC, 2019). Obesity is associated with a number of other metabolically related diseases such as Type II Diabetes, cardiovascular disease and hypertension thus increasing the rate of mortality of these individuals (reviewed in Abdelaal et al. 2017). Obesity and its associated metabolic syndromes are also associated with a number of reproductive problems, such as polycystic ovary syndrome and male infertility (Haslam & James 2005; Martins et al. 2019); in fact, links between nutrient storage and the physiological ability to carry out reproduction have been observed across many different species (mammals: reviewed in Gittleman & Thompson 1988; Bronson 2000; Schneider & Wade 2000; Schneider 2004; birds: reviewed in Martin 1987; Sibly et al. 2012; reptiles: Van Dyke et al. 2014; Lind & Beaupré 2015). Yet the relationship between obesity phenotypes such as excess nutrient storage, and reproductive behaviors, such as courtship, is not as well known. Such data could provide the beginnings of a framework to understand how the various mechanisms and traits associated with obesity shape reproductive drive and ability.

Recently, the fruit fly, \textit{Drosophila melanogaster}, has emerged as an excellent model organism to study obesity due to its high genetic conservation to humans, its ease of obtaining large sample sizes, the ease of genetic and dietary manipulations (Musselman & Kühnlein 2018). In \textit{D. melanogaster}, there exist mutants in metabolic genes that result in lean or obese phenotypes (Baker & Thummel 2007). One such gene is \textit{adipose} (\textit{adp}). The most well-characterized mutation of this gene is \textit{adp}^{60}, a 23-nucleotide deletion resulting in
increased storage of triglycerides in the fly fat body, providing an obesity phenotype (Häder et al. 2003; Suh et al. 2007; Reis et al. 2010). In addition, \textit{adp} \textsuperscript{60} mutants also are resistant to starvation and have decreased mobility compared to wildtype controls (Suh et al. 2007). While much is known about the metabolic consequences due to the loss of the \textit{adipose} gene, little is known about how the \textit{adp} \textsuperscript{60} mutation affects reproductive behaviors. Previous studies have characterized reproductive behaviors in flies with other metabolic perturbations such as wildtype flies with diet-induced obesity (Schultzhaus et al., 2017, 2018), and characterizing the courtship of the \textit{adp} \textsuperscript{60} mutant, would complement these studies.

In this study, we compare the the courtship from \textit{adp} \textsuperscript{60} male flies to wild type male flies in order to determine the effects of the \textit{adp} \textsuperscript{60} mutation on reproductive behaviors. For the purposes of this study, wildtype female flies are used throughout in order to isolate any potential effects of the \textit{adp} \textsuperscript{60} mutation and obesity on male courtship decisions. Previous studies have shown that wildtype male flies fed a high-fat diet show no effect of the resulting obesity on reproductive behaviors if fed the high-fat diet throughout development (Schultzhaus et al. 2017), but wildtype male flies do show reductions in some aspects of courtship if fed a high-fat diet only as an adult (Schultzhaus et al. 2018). In both cases, however, copulation rate is not affected. Altering the expression of the adp obesity gene may mimic one of these scenarios, or might have entirely different ramifications. For example, the \textit{adp} \textsuperscript{60} mutation might be associated with an increase in courtship behaviors resulting in an increase in copulation rate. Alternatively, it is possible that \textit{adp} \textsuperscript{60} mutants will show lower levels of reproduction compared to the wildtype, as the \textit{adp} \textsuperscript{60} mutant has also been described as having a “lethargic” phenotype (i.e., a general overall lower activity level compared to wildtype; Suh et al. 2007) and it is unclear if this lethargy phenotype is relevant to reproductive behaviors. We believe that characterizing the reproductive behaviors of \textit{adp} \textsuperscript{60} mutants will provide a better understanding of the effects of obesity on outward male sexual behavior.

\textbf{Materials and methods}

\textit{Fly husbandry}

Flies used in this study were: OreR (BL#2376) and \textit{adp} \textsuperscript{60} outcrossed into the OreR genetic background (a gift from Ronald Kuhnlein). Flies were fed cornmeal-sucrose medium (9 g \textit{Drosophila} agar, 40 g sucrose, 65 g cornmeal, 25 g Red Star whole yeast, 100 mL Karo lite corn syrup in 1200 mL dH\textsubscript{2}O). For the courtship assays, 1–2-day-old \textit{adp} \textsuperscript{60} male flies were separated from females and aged 4–5 days. These 5–6-day-old \textit{adp} \textsuperscript{60} males were then put into the courtship apparatus (see below) with 3–5-day-old wild type OreR virgin females. Thus, each fly was only used once during the course of this study.

\textbf{Courtship analysis}

One male and one female were transferred to different sides of a divider in an Aktogen\textsuperscript{®} courtship chamber by cooling the flies on ice until they slowed enough to capture and transfer with forceps. Cold exposure was never more than one minute. Once both male and female flies recovered from cooling and were walking normally in the chamber, the divider was removed allowing the two flies to interact. A video camera was placed over the chamber and all pairs were video recorded for 3 h. Four seperate chambers were always run together; two wildtype male trials were always paired with two trials of \textit{adp} \textsuperscript{60} mutants for a total of 114 wildtype male trials, and 75 \textit{adp} \textsuperscript{60} mutant trials (total numbers differ between WT and \textit{adp} \textsuperscript{60} as WT flies were used for training purposes and as a result of random incidents precluding the inclusion of flies such as escapes, deaths, or injury during trials). Lastly, all flies used were from multiple different cohorts.

Videos were uploaded to a behavioral event recorder program, Observer \textsuperscript{®}. There were three videos for flies which we know copulated that were unable to be scored due to recording or uploading errors. For the remainder of the successful videos for flies that copulated, the person scoring the behaviors did so blind to which group was being scored. The male behaviors analyzed were courtship latency, orientation, wing vibration, wing scissor, tapping, thrust, and copulation (Cobb et al. 1985). Orientation was defined as when the male oriented at any direction close to the female’s body and is typically done for the entirety for courtship. The start of orientation signaled the start of courtship and total courtship duration was measured by the start of orientation to copulation. Therefore, the start of when the divider was removed (thus allowing a male to see a female) to when orientation began was considered the latency to courtship. Wing vibration was when the male’s wings vibrated horizontally and vertically at different angles. This behavior is known to create the “courtship song” which is important in the courtship process of \textit{Drosophila} (Ewing & Bennet-Clark 1968). Wing scissor was when a fly’s wings were extended, crossed and re-crossed. Both wing scissoring and wing vibrations can be held for variable amounts of time. Tapping was when the male touched any female body part, usually sideways, with its tarsus.
Thrusting, which occurs during copulation and copulation attempts, was when the male grasped the female’s abdomen with his foretarsi, curled his abdomen downward and forward, making contact with the female’s genitalia. Copulation occurred when a male stabilized on top of the female for a considerable amount of time, typically 10 – 15 min.

Triglyceride, glycogen and protein measurements

To confirm the \textit{adp}^{60} metabolic phenotype, triglyceride, glycogen and protein levels were measured as previously described (Gingras et al. 2014). Briefly, 2 male flies were homogenized in lysis buffer (140 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.1% Triton-X 100, and 1X protease inhibitor cocktail (Roche Life Sciences)) by performing four 10-second pulses with 10-second pauses in between using a 20 kHz sonicator. Thus, one “sample” consisted of the macromolecule levels measured in two flies; 32 flies gave a N = 16 for WT, 34 flies gave a N = 17 for \textit{adp}^{60} mutants. These flies were different individuals than those used during the behavioral experiments and came from multiple cohorts.

Triglycerides and protein were measured using the Triglycerides Liquicolor Test (Stanbio) and the BCA Protein Assay Kit (ThermoScientific), respectively, according to manufacturer’s instructions. Total glucose levels were measured using the Glucose Oxidase Reagent (Pointe Scientific) after treating samples with 8 mg/mL amyloglucosidase (Sigma) in 0.2 M citrate buffer, pH 5.0 for 2 h. Free glucose was measured in samples not treated with amyloglucosidase and glycogen levels were determined by subtracting free glucose from total glucose. Triglycerides and glycogen were normalized by dividing each by the total protein concentration of each sample.

Statistical analysis

Copulation rate between fly types was tested via Chi-Square. Only individuals whose courtship resulted in copulation were used in the courtship analysis. All courtship behaviors (except latency to court) were corrected for the total duration of an individual’s courtship (i.e., the start of orientation to copulation) and then compared statistically. That is, a rate of behavior was calculated for each courtship behavior (i.e., number of times a behavior happened / time spent in courtship) to help account for the variation in length of courtship until copulation across individuals. Variables that did not conform to parametric assumptions were ln transformed to do so and tested via t-test. These variables include latency to court, number of wing scissors performed, and number of wing vibrations performed. The t statistic and degrees of freedom for the number of wing vibrations were adjusted for equal variances not being assumed for this variable. Variables that could not be transformed to conform to parametric assumptions were analyzed non parametrically using a Mann Whitney U test. These variables include time of courtship, rate of wing scissoring, rate of wing vibrations, number of thrusts, and rate of thrusts. Rates were calculated as the total time engaged in a behavior divided by the time of courtship. Tapping behavior did not occur frequently enough to warrant analysis and is therefore not presented. Both glycogen per protein and triglycerides per protein ratios met parametric assumptions and were analyzed across fly types with t-tests. The t statistic and degrees of freedom for triglycerides per protein ratios were adjusted for equal variances not being assumed. All analyses, transformations, and statistical adjustments were done using SPSS® version 25.

Results

Triglyceride and glycogen levels

Consistent with previous reports, \textit{adp}^{60} mutants had a higher triglyceride per protein ratio than wild type flies (t_{31} = 14.733, P < 0.001; Figure 1a). In addition, \textit{adp}^{60} mutants had a significantly higher glycogen to protein ratio than wild type flies (t_{25} = 5.695, P < 0.001; Figure 1b).

Copulatory and courtship behavior

The \textit{adp}^{60} mutants had a significantly lower rate of copulations relative to wild type flies (\chi^{2} = 1, N = 189 = 25.95, P < 0.001) with \textit{adp}^{60} mutants copulating at a rate of 17.3% (13/75) and wild type flies at a rate of 54.4% (62/114). Of the males who successfully copulated, there was no significant difference between fly types in total time engaged in courtship (Mann Whitney U; U = 351, Z = -0.476, N = 72, P = 0.636, Figure 2) nor latency to court (t_{70} = -1.433, P = 0.156). However, some aspects of each courtship behavior measured did differ. There was no significant difference in the total number of wing scissors (t_{70} = -1.433, P = 0.077, Figure 3a), yet wild type males did spend more time wing scissoring per total time of courtship than \textit{adp}^{60} mutants (Mann Whitney U; U = 148, Z = -3.448, N = 72, P = 0.001, Figure 3b). Wild type males also did significantly more wing vibrations than \textit{adp}^{60} mutants (t_{12.924} = -2.545, P = 0.025) and spent more time doing these wing vibrations per total time of
courtship than \( adp^{60} \) mutants (Mann Whitney U; \( U = 120, Z = -3.858, N = 72, P < 0.001 \)). There was no significant difference between fly types for number of thrusts (Mann Whitney U; \( U = 333.5, Z = -0.737, P = 0.461 \)) or the rate of thrusts per total time of courtship (Mann Whitney U; \( U = 321, Z = -0.915, P = 0.360 \)).

**Discussion**

In this study, the reproductive behaviors of the obese \( adp^{60} \) *Drosophila* mutant were characterized. The \( adp^{60} \) mutants had a lower copulation rate than the wildtype controls, consistent with the decreases in courtship behaviors observed in these animals. Therefore, it is possible that the excess nutrient storage in these mutants may be related to these behavioral differences, but also could be an independent behavioral phenotype associated with the \( adp^{60} \) mutation. Surprisingly, courtship latency, and total courtship time, between the wildtype and \( adp^{60} \) mutants were similar despite \( adp^{60} \) exhibiting lower levels of courtship behaviors during courtship. Previous studies have shown that wildtype flies fed a high fat diet also accumulate triglycerides (but not glycogen) and have similar courtship latencies as wildtype flies fed a normal diet (Birse et al. 2010; Schultzhaus et al. 2017, 2018). However, these high-fat fed flies have normal copulation frequencies (Schultzhaus et al. 2017) unlike what was observed from our current study between \( adp^{60} \) and wildtype flies (though Schultzhaus et al. (2018) argues that high-fat fed flies are less attractive than wildtype flies fed a normal diet). Therefore, some traits that are unique to the \( adp^{60} \) phenotype affect mating success.
in a way not previously reported for high fat diet flies. Such traits could include the extent to which triglycerides accumulate, the accumulation of glycogen, some combination of these two characteristics, and/or some independent behavioral phenotype associated with the \( \text{adp}^{60} \) mutation.

Despite the similar courtship times, latency to court, and thrusting behavior, \( \text{adp}^{60} \) mutants showed significantly less wing scissoring and wing vibrations. These flies had a constant availability of food but do not metabolize their triglycerides and glycogen in the same way as wild type flies (thus leading to an increased amount of each in our analysis). Potentially it is the availability and ingestion of nutrients that are involved with reproductive drive (i.e. overall courtship time and courtship latency), while the appropriate storage and usage of these sources of energy, once ingested, are involved in carrying out courtship, including the courtship song caused by the wing vibration behavior. The \( \text{adp}^{60} \) mutants are known to have a “lethargic” phenotype where their overall mobility is less than that of wild type flies (Suh et al. 2007). This phenotype may be due to this altered ability to metabolize nutrients and/or the gross obesity of these animals and could be related to the decreased courtship behaviors while reproductive drive behaviors such as latency to court remain unaffected.

It is unclear why the \( \text{adp}^{60} \) mutant contains higher levels of glycogen and triglycerides yet courted less than wild type flies, but many possibilities exist, each of which could also contribute to the overall reduced mobility phenotype of the \( \text{adp}^{60} \) mutant described above. One possibility is that the physical structure of increased fat deposits decreases wing dexterity therefore affecting courtship song however, Schultzhau et al. (2018) presented that wild type male flies fed a high-fat diet showed no significant effect on courtship song compared to wild type male flies fed a normal diet. Another possibility is that the \( \text{adp}^{60} \) mutation has additional unrealized effects on muscle activity. The \( \text{adp}^{60} \) mutation results in chronic obesity with excess fat accumulation in both the larval and adult stages of development (Häder et al. 2003; Suh et al. 2007; Reis et al. 2010). Potentially these mutants do not mobilize these fuels in the same way as wild type flies; lipid metabolism in these mutants is suspected to be different throughout development (see Teague et al. 1986 for further discussion). Any negative effect these metabolic pathways have on muscle function, in turn, could limit muscle from functioning properly and affect movement involved in courtship such as wing scissors and wing vibrations (song). Courtship song is well known to play an important role in mating within insect groups generally (reviewed in Wells & Henry 1998), and specifically within the \text{Drosophila} genus (Ewing & Bennet-Clark 1968; Spieth 1974; Hall 1994). Chakravorty et al. (2014) showed that courtship song is changed in \text{Drosophila} mutants for a major wing muscle gene, and the resulting alterations to courtship song can decrease female preference for that male. The \text{Drosophila} courtship song is known to contain important components in order for it to be successful, and therefore be a “quality” song. Specifically, there are both “pulse” and “sine” aspects to song components (von Schilcher 1976), and alterations to these aspects of song can influence female preference for the male performing the song (von Schilcher 1976; Chakravorty et al. 2014). If the \( \text{adp}^{60} \) phenotype is associated with an alteration to courtship song, it is unclear what the specific alteration might be. However, if the song is altered as suggested by the decrease in wing scissors and vibrations and the subsequent lack of copulations, it is possible that \( \text{adp}^{60} \) mutant males might have a reduced ability for generating song or might simply be choosing to decrease performing their song (due either to a female’s lack of interest and/or the males are negatively responding to their own altered song). While we cannot address which of these mechanisms is at play (or any combination thereof), they would all lead to a reduced amount of time performing song during courtship, and/or low-quality song, that could lead to an increased rejection by the female fly. In fact, the lack of courtship song from the \( \text{adp}^{60} \) mutants could help explain the low copulation rates of this group as females of various \text{Drosophila} species are known to reject males on the basis of song (Hoikkala et al. 1998; Ritchie et al. 2001).

Overall, we find that courtship behaviors are altered in the obese \( \text{adp}^{60} \) mutant in the fruit fly, \text{D. melanogaster} as this has not been described previously. More research is needed to identify the exact physiological cue that activates reproductive behavior across species and better understand the genes and physiological processes important for regulating reproductive motivation and behavior. Species such as \text{D. melanogaster} are particularly powerful in this regard as such mutants exist both naturally, such as the \( \text{adp}^{60} \) mutants used here, and can be artificially created to probe the effects of specific genes and metabolic processes on organismal reproduction to better understand the reproductive ramifications the obesity phenotype and its associated traits.
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Disclosure statement

No potential conflict of interest was reported by the authors.

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