Journal of Insect Physiology 107 (2018) 152–156

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Dynamic changes in ejaculatory bulb size during *Drosophila melanogaster* aging and mating

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ARTICLE INFO

Keywords:
Drosophila melanogaster
Ejaculatory bulb
Mating
Aging
Seminal fluid proteins
PEBme

ABSTRACT

The ejaculatory bulb of *Drosophila melanogaster* males produces proteins and pheromones that play important roles in reproduction. This tissue is also the final mixing site for the ejaculate before transfer to the female. The ejaculatory bulb's dynamics remain largely unstudied. By microscopy of the ejaculatory bulb in maturing adult males, we observed that the ejaculatory bulb expands in size as males age. Moreover, we document that when males mate, their ejaculatory bulb expands further as ejaculate transfer begins, and then contracts halfway through the course of mating as ejaculate transfer finishes. Although there is some male-to-male variation in the timing of these changes, ultimately the tissue changes in a predictable pattern that gives insight into the active mating process in *Drosophila*.

1. Introduction

Seminal fluid molecules are essential for normal and successful reproduction, making it of interest to understand the biology of the tissues that produce or transmit them. For example, studies in *Drosophila melanogaster* have shown that the male's accessory glands, which produce many peptides and proteins that cause critical post-mating changes in females (reviewed in Avila et al., 2011), contain two secretory cell types (around a lumen) with different structure, synthetic characteristics, and functions (Bertram et al., 1992; Bairati, 1968; Gligorov et al., 2013; Sitnik et al., 2016; Leiblich et al., 2012; Minami et al., 2012).

Another important secretory tissue in the male *D. melanogaster* reproductive tract is the ejaculatory bulb (EB) (reviewed in Avila et al. 2015). This tissue is at the most distal end of the male's reproductive tract (closest to the outside of the male), a position at which a number of Diptera and other insects have broadenings or specializations of the ejaculatory duct (a few examples are described by Patterson, 1943; Bjork et al., 2007; Herrera-Cruz et al., 2017; Chiang and Chiang, 2016; Ramamurthi, 1950; Kamimura, 2008). The EB is the last part of the reproductive tract through which other ejaculate components pass on their way into the female. Its secretions, added to the seminal fluid and transferred to females during mating, include proteins that coagulate to form the mating plug that retains the ejaculate in the female (and may facilitate sperm movement). One EB protein, the fluorescent protein PEBme (Ludwig et al., 1991; Lung, 2001), has sequence features suggesting a structure that can mediate this coagulation, and its removal from males interferes with mating plug formation and thus retention of ejaculate in mated females (Avila et al., 2015). Another EB protein, PEB2, regulates female attractiveness shortly after mating (Bretman et al., 2009). The EB also provides the hydrocarbon *cis*-vaccenyl acetate (cVA) which, after transfer to females, contributes to the pheromone blend that makes females unattractive to males for several hours post-mating (Laturney and Billeter 2016). In addition to generating these important molecules, the EB is also thought to pump the ejaculate into females during mating.

In 1968, Bairati observed through structural and ultrastructural analysis that the EB is composed of a receptive apparatus that appears to control bulb compression (Bairati, 1968). The ejaculatory bulb was described as "kidney bean shaped," with four cylindrical "horns" that extend on all sides of the ejaculatory bulb's central depression Fig. 1) (Bairati, 1968). We wondered whether the accumulation and ejaculation of EB contents might impact the tissue's shape during development and mating. Accordingly, we examined the morphology of the EB as male flies mature and mate. We report here that the EB consists of a lumen surrounded by cells, and further surrounded by muscles, and that the tissue expands as the males age and synthesize EB secretions. We also report that the EB expands further, and then contracts, during mating, presumably reflecting ejaculate accumulation and entry into, and then release from, this tissue.

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https://doi.org/10.1016/j.jinsphys.2018.04.005
Received 12 February 2018; Received in revised form 6 April 2018; Accepted 6 April 2018
Available online 07 April 2018
0022-1910/

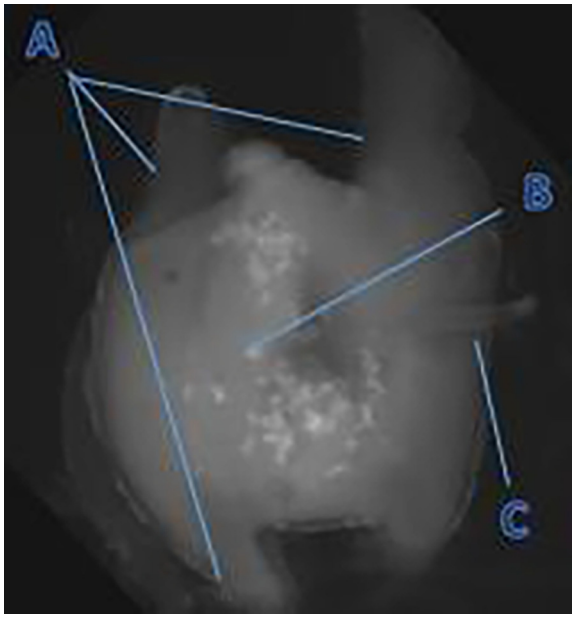

Fig. 1. A normal ejaculatory bulb from an unmated 3-day-old Canton S male. Labels are based on Bairati (1968). "A" points to 3 of the 4 "horns"; there is one horn at each of the four corners of the EB. B shows a point in the bulbar cavity, the rounded region in the center, which appears semicircular from the side. Bright autofluorescent particles are seen in this cavity, as discussed in the text. C marks the sclerite handle which, through a series of fibers, connects the two sides.

2. Materials and methods

2.1. Fly strains

Unless otherwise noted, all flies used in this study were from the Canton S strain. Flies were raised on yeast-glucose agar medium at 23 °C with a 12:12 light:dark photoperiod. Tubulin-Gal4; UAS-MHC-GFP flies (Hughes and Thomas, 2007) whose myosin heavy chain is labeled with green fluorescent protein, were used to visualize the muscles surrounding the ejaculatory bulb.

2.2. Mating

Matings were carried out with 3-day-old virgin males, and 3–5 day old virgin females, all previously aged in single-sex cultures. One fly of each sex was placed into a cylindrical glass vial. Pairs were observed to note the initiation of mating; mating typically lasted ~17 min. To measure changes in EB size during mating, flies were collected at the following time points: unmated, and at 2 min, 5 min, 10 min, 25 min, 1 h, and 3 h after the start of mating (ASM). For the 2 min, 5 min and 10 min ASM time-points, mating pairs that were separated by vigorous centrifugal motion of the vial; for the 3 latest time-points, the flies had uncoupled naturally, and the female was removed from the vial immediately after the uncoupling. Uncoupled males were aspirated individually into microcentrifuge tubes, flash frozen in liquid nitrogen, and stored at -80 °C. Sample sizes ranged from 6 to 54 flies per time-point, with an average of 20.

2.3. Dissection & microscopy

Using stainless steel forceps, we dissected each male in 1× PBS, under a dissection microscope. The EB and one wing from each fly were mounted on a glass slide with two drops of PBS, placed under a glass coverslip, and examined using a Leica DM 500B fluorescence microscope. Under these conditions, the EB is slightly flattened, and its width (taken left-to-right across the vertical middle of EBs oriented as in Fig. 1 is 0.26 mm. To measure EB size, we photographed the flattened tissue under 4× magnification, and used the freehand selections tool of Image J to trace the outline of one half of the EB (a line was drawn vertically through the center of the bilaterally symmetrical EB, and the most clearly visible half was traced, as both halves were not always fully visible or otherwise measurable). The area of the traced shape was determined with Image J, and measuring a straight line from the center of the anterior edge of the wing to the center of the posterior edge of the wing. For normalization, we measured the width of one wing of the same fly, using the straight lines tool in Image J. Results were analyzed statistically using ANOVA, in RStudio. For all experiments, dissections, microscopy, and measurements were performed blind as to treatment group. To visualize MHC-GFP staining, EB dissected from tub > MHC-GFP males were excited with 500 nm wavelength light from a mercury lamp, and visualized with an I3 filter under a Leica DM 500B fluorescence microscope. Autofluorescence of PEBme in EBs or mated females was examined under UV illumination on the Leica DM 500B microscope, and EBs and wings were photographed under the UV filter.

2.4. DAPI staining

DAPI (4′, 6-diamidine-2-phenylindole) staining was done as described in Wu and Luo (Wu and Luo, 2007), with minor modifications for dissection method, and the use of a final DAPI concentration of 1.75 μg DAPI/μL PBS.

3. Results and discussion

3.1. The ejaculatory bulb contains a lumen and is surrounded by muscle

Bairati (1968) posited that the ejaculatory bulb had a lumen full of seminal components and was able to compress during ejaculation, to pump the ejaculate into the female during mating. But the cellular and cell-type structure of this tissue had not been investigated. We stained EBs with DAPI to visualize the cellular composition. We saw that the center of the EB is a bulbar cavity devoid of cells (Fig. 2B), consistent with Bairati's view that the EB has a lumen to hold ejaculate (Bairati, 1968). To determine whether the EB is surrounded by muscles to facilitate contraction, as in other reproductive tract tissues (Norville et al., 2010), we examined MHC-GFP distribution on the EB of transgenic males. We observed that muscle surrounds the EB (Fig. 2A), with especially high density around the bulbar cavity.

Ejaculatory bulb secretions that are transferred to females include

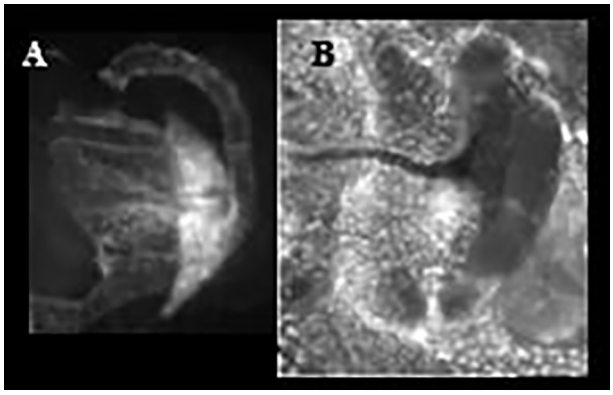

Fig. 2. The ejaculatory bulb contains a lumen, and is surrounded by muscle. A) Muscles surrounding the EB are visualized with MHC-GFP in this EB of a mated 3-day-old tub > MHC-GFP male. B) A DAPI-stained EB, from a 3-day-old unmated Canton S male. The orientation of this EB is similar to that shown in panel A. Note the acellular lumen (unstained by DAPI) and the duct leading out of the EB.