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Study of Sperm Utilization in Female *Drosophila melanogaster* of *bdp* and *btx* Strains

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Abstract

This study investigates the patterns of sperm utilization in female Drosophila melanogaster of the *bdp* and *btx* strains through sequential mating with multiple males. The research was conducted as a descriptive observational study without experimental manipulation. Females of each strain were crossed with four different males in a specific order, with a two-day interval between each mating. F1 offspring phenotypes were recorded daily over a six-day period following the final mating. Data were analyzed descriptively and supported by somatic chromosome reconstruction to identify the male origin of each offspring. The results indicate that sperm utilization in D. melanogaster females occurs in both random and non-random patterns. Non-random utilization was observed when offspring were produced exclusively from the first male, with no contribution from subsequent males. For example, in the ♀*btx* × ♂*N* cross, only *N*♀ and *N*♂ offspring were produced, indicating that sperm from the first male was preferentially used. In contrast, random sperm utilization was evident in later matings, where offspring from multiple males appeared, suggesting that previously stored sperm remained viable and were used alongside or instead of sperm from later matings. These findings highlight the complexity of post-mating sexual selection in D. melanogaster, particularly the role of female sperm storage and utilization mechanisms. The ability of females to either favor the sperm of a particular male or mix sperm from several males may provide evolutionary advantages in terms of genetic diversity and offspring fitness. This study contributes to a better understanding of reproductive strategies and sperm competition in insects, with potential implications for broader studies in evolutionary biology and genetics.

Keywords: *Drosophila melanogaster*; sperm utilization; sequential mating; sperm competition; chromosome analysis.

Abbreviations: black dumpy wings (*bdp*); black taxi (*btx*); dumpy wings (*dp*); First filial generation (F1); Normal (wild-type) strain of *Drosophila melanogaster* (*N*); taxi (*tx*)

INTRODUCTION

One of the characteristics of living organisms is reproduction, which serves the purpose of preserving the species. Reproduction can occur either sexually or asexually. Sexual reproduction typically involves mating. One example of an animal that reproduces sexually through mating is *Drosophila melanogaster*. *D. melanogaster* is a favored model organism in genetic research due to several advantages, including its small size, complex anatomical structure, ease of handling, ability to be easily bred in the laboratory, simple and inexpensive breeding conditions, short life cycle, and the large number of described mutants whose information is readily available. Additionally, it has a small number of chromosomes and features giant chromosomes in the salivary glands of mature larvae (King, 1965).

Mating in *D. melanogaster* begins once the flies reach sexual maturity. In males, sexual maturity is marked by the ability to produce and release sperm, while in females it is indicated by the ability to ovulate ootids. In females, sperm from the male is stored in two seminal receptacles and in the paired spermathecae, the latter being the organ for long-term sperm storage (Clark et al., 1994). Furthermore, Clark et al. (1994) state that female *D. melanogaster* exhibit efficient sperm utilization. Their study also found that remating increases fertility in females. When a female mates again with another male, there is a phenomenon where the sperm from the second male is preferentially used. This is undoubtedly influenced by factors that regulate when and how sperm is stored. One such factor determining a female's receptivity to remate is a decrease in the amount of stored sperm (Clark et al., 1994).

Sperm utilization in fertilization can be random or non-random. Random utilization occurs when offspring from the first male appear in the progeny of subsequent matings with the second, third, or fourth male—in other words, when earlier sperm continue to contribute to later crosses. Non-random utilization occurs when offspring from the first male do not appear in subsequent crosses with other males, meaning that each cross produces only the offspring phenotype associated with the male used in that particular mating.

MATERIALS AND METHODS

Research Design

This study is a descriptive observational study, as no specific treatments were applied. The research was conducted by sequentially mating ♀*bdp* with ♂*N*, ♂*bdp*, ♂*b*, and ♂*dp*, and ♀*btx* with ♂*N*, ♂*btx*, ♂*b*, and ♂*tx* in four different mating schemes, in order to examine sperm utilization in ♀*bdp* and ♀*btx*. The data collected were analyzed descriptively using somatic chromosome reconstruction analysis.

Population and Sample

The population in this study consisted of *D. melanogaster* obtained from the Genetics Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Malang. The sample used included *D. melanogaster* of the following strains: *bdp*, *dp*, b, *btx*, *tx*, and *N*.

Procedure

Medium Preparation

The preparation of the medium began with weighing the main ingredients: rajamala banana, cassava tapai, and palm sugar in a 7:2:1 ratio. The banana and tapai were blended with water until smooth. The mixture was then poured into a pot, combined with sliced palm sugar, and cooked over a stove for 45 minutes. Once cooked, the mixture was poured hot into jam jars and immediately sealed with sponge stoppers. The jars were then cooled in a container filled with water. After cooling, the lid was opened to add 5–7 yeast granules and a piece of pupariation paper, then resealed with sponge.

Ampule Preparation

Pupae that had turned dark were collected from *D. melanogaster* stock cultures of each strain using a fine brush. These pupae were placed into jars containing the prepared medium, then sealed with a piece of sponge or foam.

Mating Procedure

In the mating stage, two female genotypes were used: ♀*bdp* and ♀*btx*. Each was crossed using four different mating schemes.

For ♀*bdp*, the mating schemes were as follows:

- Type I: ♀*bdp* was first mated with ♂*N*. After 2 days, she was transferred to a new medium and mated with ♂*bdp* while ♂*N* was removed. Two days later, ♂*bdp* was removed and ♀*bdp* was crossed with ♂*b*, followed by ♂*dp* after ♂*b* was removed. Two days after the final male was removed, ♀*bdp* was moved to fresh medium without further mating and released once pupae were observed. F1 phenotypes were recorded and counted from days 0–6. The process was repeated three times.
- Type II: ♀*bdp* was first mated with ♂*bdp*, then sequentially with ♂*b*, ♂*dp*, and ♂*N*, each with a 2-day interval and replacement of the previous male. After mating with the final male, ♀*bdp* was transferred to fresh medium without mating and released after pupae appeared. F1 offspring were observed and recorded over six days, and the procedure was repeated three times.
- Type III: ♀*bdp* was mated with ♂*b*, ♂*dp*, ♂*N*, and ♂*bdp*. The process of transferring to new media and replacing males every 2 days was the same. Phenotypic observations were carried out from day 0 to day 6 and repeated three times.
- Type IV: ♀*bdp* was mated with ♂*dp*, followed by ♂*N*, ♂*bdp*, and finally ♂*b*. Each male was removed after 2 days, with the female transferred to fresh media. She was released after pupae were observed. F1 phenotypes were recorded daily for six days, and the procedure was repeated three times.

For ♀*btx*, the same procedure was used with different male partners: ♂*N*, ♂*btx*, ♂*b*, and ♂*tx*.

- Type I: ♀*btx* was sequentially mated with ♂*N*, ♂*btx*, ♂*b*, and ♂*tx*, each with a 2-day interval and replacement of the previous male. After all matings, ♀*btx* was moved to fresh medium and released once pupae appeared. F1 phenotypes were observed for six days, and the procedure was repeated three times.
- Type II: The mating order was ♂*btx*, ♂*b*, ♂*tx*, and ♂*N*. The same interval, transfer, and release procedures were followed as in Type I.
- Type III: ♀*btx* was mated in sequence with ♂*b*, ♂*tx*, ♂*N*, and ♂*btx*. F1 phenotypes were recorded from day 0 to day 6, and the procedure was repeated three times.
- Type IV: The mating order was ♂*tx*, ♂*N*, ♂*btx*, and ♂*b*. Transfers to fresh media occurred every 2 days, with each male removed before the next. Observations and recordings of F1 phenotypes were done for six days and repeated three times.

RESULTS AND DISCUSSION

Result

The characteristics of *D. melanogaster* strains **(Figure 1)** used in this study are as follows:

a. ***Normal strain (N)***
  - Bright red eyes
  - Smooth eye facets
  - Light brown body color
  - Wings cover the body and extend beyond body length

b. Black strain (b)
- Red eyes
- Smooth eye facets
- Black body color
- Wings longer than the body

c. Taxi strain (tx)
- Red eyes
- Smooth eye facets
- Light brown body color
- Wings longer than the body and lifted upward

d. Dumpy wings strain (dp)
- Red eyes
- Smooth eye facets
- Light brown body color
- Wings are shortened and notched

e. Black taxi strain (btx)
- Red eyes
- Smooth eye facets
- Black body color
- Wings longer than the body and lifted upward

f. Black dumpy wings strain (bdp)
- Red eyes
- Smooth eye facets
- Dark brownish-black body color
- Notched wings

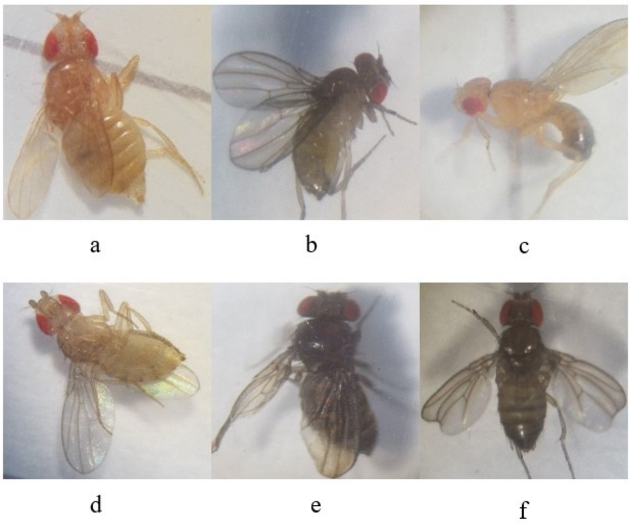

Figure 1. The characteristics of *D. melanogaster* strains: a) Normal strain (N), b) Black strain (b), c) Taxi strain (tx), d) Dumpy wings strain (dp), e) Black taxi strain (btx), and f) Black dumpy wings strain (bdp).

Table 1. Phenotypic Count Data.

No	Type	Cross	F1	Sex	Replicate			Σ	Total	Note
					1	2	3			
1	I	♀*bdp*><♂N			-	-	-	-		
		♀*bdp*><♂*bdp*			-	-	-	-		
		♀*bdp*><♂*b*			-	-	-	-		
		♀*bdp*><♂*dp*			-	-	-	-		
2	II	♀*bdp*><♂*bdp*	*bdp*	♂	8	15	-	23	43	Non- Random
			bdp	♀	11	9	-	20		
		♀*bdp*><♂*b*	*bdp*	♂	11	0	-	11	17	Random
			bdp	♀	6	0	-	6		
			b	♂	9	0	-	9	17	
			b	♀	8	0	-	8		
		♀*bdp*><♂*dp*	*bdp*	♂	7	0	-	7	9	Random
			bdp	♀	2	0	-	2		
			dp	♂	6	0	-	6	12	
			dp	♀	6	0	-	6		
			b	♂	5	0	-	5	10	
			b	♀	5	0	-	5		
		♀*bdp*><♂N	*bdp*	♂	4	0	-	4	7	Random
			bdp	♀	3	0	-	3		
			dp	♂	3	0	-	3	11	
			dp	♀	8	0	-	8		
			b	♂	5	0	-	5	9	
			b	♀	4	0	-	4		
			N	♂	5	0	-	5	10	
			N	♀	5	0	-	5		
3	III	♀*bdp*><♂*b*			-	-	-	-	-	
		♀*bdp*><♂*dp*			-	-	-	-	-	
		♀*bdp*><♂N			-	-	-	-	-	
		♀*bdp*><♂*bdp*			-	-	-	-	-	
4	IV	♀*bdp*><♂*dp*			-	-	-	-	-	

No	Type	Cross	F1	Sex	Replicate			Σ	Total	Note
					1	2	3			
		♀*bdp*><♂N			-	-	-	-	-	
		♀*bdp*><♂*bdp*			-	-	-	-	-	
		♀*bdp*><♂b			-	-	-	-		
5	V	♀*btx*><♂N	N	♂	8	-	-	8	13	Non- Random
			N	♀	5	-	-	5		
		♀*btx*><♂*btx*	*btx*	♂	7	-	-	7	10	Random
			btx	♀	3	-	-	3		
			N	♂	6	-	-	6	6	
		♀*btx*><♂*b*	*b*	♂	2	-	-	2	9	Random
			b	♀	7	-	-	7		
			N	♂	1	-	-	1	3	
			N	♀	2	-	-	2		
		♀*btx*><♂*tx*	N	♂	10	-	-	10	12	Random
			N	♀	2	-	-	2		
			b	♂	1	-	-	1	7	
			b	♀	6	-	-	6		
6	VI	♀*btx*><♂*btx*	*btx*	♂	11	-	-	11	23	Non-Random
			btx	♀	12	-	-	12		
		♀*btx*><♂*b*	*b*	♂	25	-	-	25	44	Random
			b	♀	12	-	-	12		
			N	♂	4	-	-	4		
			N	♀	3	-	-	3		
		♀*btx*><♂*tx*	*b*	♂	9	-	-	9	35	Random
			b	♀	19	-	-	19		
			tx	♂	2	-	-	2		
			tx	♀	5	-	-	5		
		♀*btx*><♂N	*b*	♂	5	-	-	5	14	Random
			b	♀	9	-	-	9		
			btx	♂	4	-	-	4	7	
			btx	♀	3	-	-	3		
			tx	♂	5	-	-	5	11	
			tx	♀	6	-	-	6		
			N	♂	6	-	-	6	10	
			N	♀	4	-	-	4		
7	VII	♀*btx*><♂*b*	*b*	♂	12	-	-	12	23	Non-Random
			b	♀	11	-	-	11		
		♀*btx*><♂*tx*	*tx*	♂	9	-	-	9	21	Random
			tx	♀	12	-	-	12		
			b	♂	13	-	-	13	15	
			b	♀	8	-	-	8		
		♀*btx*><♂N	N	♂	8	-	-	8	15	Random
			N	♀	7	-	-	7		
			tx	♂	10	-	-	10	19	
			tx	♀	9	-	-	9		
			b	♂	7	-	-	7	12	
			b	♀	5	-	-	5		
		♀*btx*><♂*btx*	*btx*	♂	8	-	-	8	15	Random
			btx	♀	7	-	-	7		
			tx	♂	8	-	-	8	14	
			tx	♀	6	-	-	6		
			b	♂	6	-	-	6	14	
			b	♀	8	-	-	8		
			N	♂	9	-	-	9	14	
			N	♀	5	-	-	5		
8	VIII	♀*btx*><♂*tx*	*tx*	♂	7	5	15	27	39	Non-Random
			tx	♀	2	3	7	12		
		♀*btx*><♂N	*tx*	♂	5	20	7	32	69	Random
			tx	♀	7	21	9	37		
			N	♂	2	5	13	20	37	
			N	♀	2	7	8	17		
		♀*btx*><♂*btx*	*btx*	♂	2	8	8	18	45	Random
			btx	♀	8	12	7	27		

No	Type	Cross	F1	Sex	Replicate 1	Replicate 2	Replicate 3	Σ	Total	Note
			tx	♂	1	0	9	10	18	
			tx	♀	1	0	7	8		
			N	♂	0	0	8	8	16	
			N	♀	0	0	8	8		
		♀*btx*><♂b	*btx*	♂	5	1	6	12	25	Random
			btx	♀	3	0	10	13		
			tx	♂	2	0	6	8	19	
			tx	♀	1	0	10	11		
			b	♂	3	5	8	16	26	
			b	♀	1	2	7	10		
			N	♂	1	0	8	9	18	
			N	♀	0	0	9	9		

Chromosome Reconstruction Analysis

a. *Somatic Chromosome Reconstruction ♀bdp*

1) ♀bdp X ♂N

P $\frac{bdp}{bdp} \text{X} \frac{b^+dp^+}{b^+dp^+}$

G $b\,dp$ $\quad b^+dp^+$

F1 $\frac{b^+dp^+}{bdp}$ (N)

2) ♀bdp X ♂*bdp*

P $\frac{bdp}{bdp} \text{X} \frac{bdp}{bdp}$

G bdp $\quad bdp$

F1 $\frac{bdp}{bdp}$ (*bdp*)

3) ♀*bdp* X ♂*b*

P $\frac{bdp}{bdp} \text{X} \frac{bdp^+}{bdp^+}$

G bdp $\quad bdp^+$

F1 $\frac{bdp^+}{bdp}$ (*b*)

4) ♀*bdp* X ♂*dp*

P $\frac{bdp}{bdp} \text{X} \frac{b^+dp}{b^+dp}$

G bdp $\quad b^+\,dp$

F1 $\frac{b^+dp}{bdp}$ (*dp*)

b. *Somatic Chromosome Reconstruction* ♀ *btx*

1) ♀*btx* X ♂ N

P $\frac{btx}{btx} \text{X} \frac{b^+tx^+}{b^+tx^+}$

G btx $\quad b^+tx^+$

F1 $\frac{b^+tx^+}{btx}$ (N)

2) ♀*btx* X ♂*btx*

P $\frac{btx}{btx} \text{X} \frac{btx}{btx}$

G btx $\quad btx$

F1 $\frac{btx}{btx}$ (*btx*)

3) ♀*btx* X ♂*b*

P $\frac{btx}{btx} \text{X} \frac{btx^+}{btx^+}$

G btx $\quad btx^+$

F1 $\frac{btx}{btx^+}$ (*b*)

4) ♀*btx* X ♂*tx*

P $\frac{btx}{btx} \text{X} \frac{b^+tx}{b^+tx}$

G btx $\quad b^+\,tx$

F1 $\frac{btx}{b^+tx}$ (*tx*)

Crosses in *bdp* Female Individuals

Based on the observations, data from cross type I, in which a *bdp* female was mated with a first male *N*, showed that the resulting F1 offspring had the *N* phenotype. Cross type II, between a *bdp* female and a first male *bdp*, produced F1 offspring with the *bdp* phenotype. Cross type III, between a *bdp* female and a first male *b*, resulted in F1 offspring with the *b* phenotype. Cross type IV, with a first male *dp*, produced F1 offspring with the *dp* phenotype. These results indicate that all F1 offspring from crosses between *bdp* females and the first males were non-random, matching the chromosome reconstruction results. For cross type I with a second male *bdp* (♀*bdp* × ♂*bdp*), no data were obtained as the cross had not yet been performed. Cross type II (♀*bdp* × ♂b) yielded F1 offspring with both *bdp* and *b* phenotypes. The number of individuals with *b* and *bdp* phenotypes was equal. Cross types III and IV, with second males *dp* and N (♀*bdp* × ♂*dp* and ♀*bdp* × ♂N), had no data available yet.

Cross type I with a third male b (♀*bdp* × ♂*b*) also had no available data. Cross type II (♀*bdp* × ♂*dp*) produced F1 offspring with *bdp*, *dp*, and *b* phenotypes. Among them, the *dp* phenotype was more frequent than the *bdp* and *b*. Cross types III and IV (♀*bdp* × ♂*N* and ♀*bdp* × ♂*bdp*) also had no data yet. Cross type I with a fourth male *dp* (♀*bdp* × ♂*dp*) had no data. Cross type II (♀*bdp* × ♂*N*) resulted in F1 offspring with *bdp*, *dp*, *b*, and *N* phenotypes. The *dp* phenotype appeared most frequently. Cross types III and IV (♀*bdp* × ♂*bdp* and ♀*bdp* × ♂b) had no data as the crosses had not been performed.

Overall, observations from crosses between *bdp* females and second, third, and fourth males showed that the F1 offspring did not only exhibit the phenotypes of the male parent involved in the cross. This contradicts the F1 phenotypes predicted by chromosome reconstruction, suggesting that sperm usage was random. Random sperm usage is evident when offspring resulting from the first male also appear in crosses with second, third, or fourth males.

Crosses in *btx* Female Individuals

From the observations, in cross type V (♀*btx* × ♂*N*), the F1 offspring had the *N* phenotype. Cross type VI (♀*btx* × ♂*btx*) resulted in *btx* phenotype. Cross type VII (♀*btx* × ♂*b*) produced *b* phenotype. Cross type VIII (♀*btx* × ♂*tx*) yielded *tx* phenotype. These results indicate that all F1 offspring from *btx* females crossed with the first males were non-random and matched the reconstructed chromosome outcomes.

Cross type V (♀*btx* × ♂*btx*) with a second male resulted in F1 offspring with *btx* and *N* phenotypes. Cross type VI (♀*btx* × ♂*b*) produced *b* and *N* phenotypes, with more *b* individuals than *N*. Cross type VII (♀*btx* × ♂*tx*) resulted in *tx* and *b* phenotypes, with equal numbers of each. Cross type VIII (♀*btx* × ♂*N*) resulted in *tx* and *N* phenotypes, with *tx* being more frequent. In cross type V with a third male *b* (♀*btx* × ♂*b*), the F1 offspring had *b* and *N* phenotypes. Cross type VI (♀*btx* × ♂*tx*) resulted in *b* and *tx* phenotypes, with more *b* than *tx*. Cross type VII (♀*btx* × ♂*N*) produced *N*, *tx*, and *b* phenotypes, with *tx* being the most frequent. Cross type VIII (♀*btx* × ♂*btx*) yielded *btx*, *tx*, and *N* phenotypes, with *btx* being dominant.

Cross type V with a fourth male *tx* (♀*btx* × ♂*tx*) yielded *b* and *N* phenotypes. Cross type VI (♀*btx* × ♂*N*) resulted in *b*, *btx*, *tx*, and *N* phenotypes, with *b* being the most frequent. Cross type VII (♀*btx* × ♂*btx*) also resulted in *btx*, *tx*, *b*, and *N* phenotypes, with *btx* being dominant. Cross type VIII (♀*btx* × ♂*b*) produced the same set of phenotypes, with *b* being the most frequent. All cross data between *btx* females and the second, third, and fourth males show that F1 offspring did not consist solely of the male parent's phenotype, again contradicting the chromosome reconstruction results. This indicates random sperm usage, as offspring from the first male appeared in crosses with subsequent males.

Discussion

Sperm Utilization in *D. melanogaster btx* and *bdp* Female Individuals

Analysis of the data showed that all crosses between ♀*bdp* and ♀*btx* females with the first male produced F1 offspring matching the chromosome reconstruction. However, crosses with the second, third, and fourth males yielded F1 phenotypes inconsistent with the expected results, suggesting that sperm utilization in those cases was random. In the first mating, fertilization was non-random because the female had only mated with one male, and thus only one type of sperm was used. In subsequent matings, the appearance of phenotypes associated with the first male's sperm indicates random sperm utilization. This phenomenon is known as sperm displacement or sperm precedence.

Sperm displacement occurs when sperm from the first male is physically moved or displaced within the female's sperm storage organs (seminal receptacle and spermatheca) by sperm from a subsequent male. This reduces the likelihood that the first male’s sperm will be used for fertilization. Sperm precedence means that sperm from the second male is more likely to fertilize eggs due to the influence of his seminal fluid. This fluid can suppress the motility and capacitation of the first male's sperm, giving the second male's sperm an advantage. According to Clark et al. (1994), sperm precedence involves two main aspects: (1) physically blocking or displacing earlier sperm, and (2) resisting displacement after copulation.

In this study, a single *D. melanogaster* female was sequentially mated with four different males every two days. This interval aligns with the female's receptivity — her willingness to accept a new mate. After mating, females generally show decreased receptivity due to behavioral and physiological changes, including reduced attractiveness and reluctance to remate (Grillet, 2006; Singh, 2002). These post-mating changes are driven by copulation effects (where male seminal proteins suppress female receptivity to prevent sperm competition) and sperm effects (linked to sperm quantity and usage). The nervous system female receptivity, and stimulation from sperm movement in storage organs influences behavior.

Importantly, *D. melanogaster* females do not wait for stored sperm to be depleted before remating. Thus, sperm from the first male remains present during later matings, enabling sperm competition.

Competition arises as new sperm enters storage and competes with earlier sperm. Several factors influence remating frequency and sperm competition, such as sperm count, seminal fluid composition, nutrition, egg laying, testes size, and body size. Specific genes and proteins are known to play roles in sperm competition. In *D. melanogaster*, sperm from later males is generally more successful. Seminal fluid from subsequent males can suppress capacitation of earlier sperm, without necessarily removing them from storage. Sperm storage organs in females play a key role in prioritizing sperm usage or preventing fertilization by earlier sperm. Male accessory gland proteins are critical in this process (Adrianne et al., 2008). Genes expressed in the spermatheca are known to encode serine proteases, which interact with male proteins and influence fertilization outcomes.

These proteases may regulate the internal environment of the spermatheca similarly to human prostate-specific antigen (PSA), which aids semen liquefaction. Other genes, such as trehalase, may help maintain sperm by regulating sugar metabolism. In short, interactions between male seminal proteins and female reproductive proteins determine which sperm succeed. In this study, such interactions likely resulted in sperm from second or later males being favored, which explains why their phenotypes dominated in the F1 generation.

CONCLUSIONS

Based on the discussion above, a preliminary conclusion can be drawn that sperm utilization in *D. melanogaster* females of the *bdp* and *btx* strains occurs both randomly and non-randomly. Non-random utilization is observed in the first mating; for example, in the cross ♀*btx* × ♂N (first male), the resulting F1 offspring consisted only of N♀ and N♂ phenotypes. In contrast, random sperm utilization appears in the second, third, and fourth matings — in other words, in crosses involving subsequent males.

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