INVESTIGATING THE FEMALE'S ROLE IN SPERM COMPETITION IN *DROSOPHILA MELANOGASTER*

A Thesis

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ABSTRACT

The process of fertilization involves many interactions between males, females, and their gametes. This is even more complex in cases of multiple mating, such as in *Drosophila melanogaster*, as the presence of ejaculates from multiple males and the female's ability to store sperm presents the opportunity for sperm competition to occur. In *D. melanogaster*, male-derived seminal fluid proteins are known to influence various post-mating responses in the female including sperm competition outcomes. While studies have shown that female genotype is also important for sperm competition outcome, the mechanisms underlying the female's contribution to the success of a particular male's sperm are less understood.

To begin to examine the female's role in sperm competition, we took two approaches in *D. melanogaster*: First, we used RNAi knockdown of candidate genes to assess the impact of decreased expression of these genes on sperm competition outcomes. We found that of 29 candidate genes tested, 10 affect sperm competition outcomes when knocked down in females. Second, we used fly lines mutant for neuromodulators to assess sperm storage outcomes from two different types of males mated sequentially. We found that the neuromodulators octopamine and tyramine may influence relative sperm storage of sperm from competing males. Collectively, results from these experiments provide a clearer picture of the genes and mechanisms involved in the female control of sperm competition outcomes and sperm dynamics.

BIOGRAPHICAL SKETCH

Simone White received a B.A. in Biological Sciences from Binghamton University, Binghamton, NY in 2011.

This thesis is dedicated to my parents and sister who always unconditionally support and love me. I would not be where I am today without the three of you. You will always be the most important people in my life, and for that I am extremely privileged and grateful.

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INTRODUCTION

In *Drosophila melanogaster*, females often mate with and store sperm from multiple males. Female *D. melanogaster* can store sperm in two types of sperm storage organs, a single seminal receptacle and a pair of spermathecae. Storage of sperm from multiple males allows sperm competition to occur inside the female's reproductive tract (Milkman & Zeitler 1974; Prout & Bundgaard 1977; Imhof et al. 1998). The ability of *Drosophila* females to store sperm, along with the numerous tools available for study of *Drosophila* genes make them a particularly good model to study sperm competition.

The outcome of sperm competition depends on a network of complex interactions between male and female molecules. However, males and females often have different reproductive interests, leading to sexual conflict. For example, in systems where females mate multiply, males are often in direct competition with other males (Parker 1970). This may result in males having a damaging effect on females through seminal protein toxicity (Chapman et al. 1995; Wigby & Chapman 2004; Mueller et al. 2007) and through inhibiting a female's likelihood of remating. However, the reproductive interests of females are to produce the most highly fit offspring. So, for example, it is often in the female's interest to mate multiply, so as to increase the fitness of progeny, while at odds with the male's strategy to inhibit remating by females. This conflict from different evolutionary goals of males and females suggests that the mechanisms behind sperm competition may be distinct in both sexes.

D. melanogaster males have been shown to influence a variety of post-mating characteristics through transfer of seminal fluid proteins. These characteristics include sperm competition (Clark et al. 1995; Fiumera et al. 2005, 2006, 2007; Chow et al. 2010; Greenspan & Clark 2011). Moreover, male genotype and seminal fluid proteins have been shown to be

important for determining the outcome of sperm competition (Clark et al. 1995, 1999; Fiumera et al. 2005, 2007; Chow et al. 2010; Civetta et al. 2008).

The female's role sperm competition is much less understood. Female genotype has been shown to affect sperm competition outcome (Clark & Begun 1998; Clark et al. 1995, 1999; Chow et al. 2010), as do male X female genotype interactions (Clark & Begun 1998; Clark et al. 1999; Chow et al. 2010). However, there is limited knowledge about the individual genes and processes involved in the female contribution to sperm competition and dynamics. Females may influence sperm competition in several different ways. For example, females could modulate muscular contractions in the reproductive tract that move sperm into and out of storage through nervous system inputs, or spermathecal secretions promoting sperm viability while sperm are in storage could influence sperm competition outcomes. Genes expressed in the female reproductive tract might produce proteins that influence sperm competition outcomes (Swanson et al. 2004; Mack et al. 2006; Allen & Spradling 2008; Kapelnikov et al. 2008; Prokupek et al. 2008, 2009, 2010). Only two genes when altered in female *D. melanogaster*, *Sex Peptide Receptor* (*SPR*; Chow et al. 2010) and *Neprilysin 2* (*Nep2*; Sitnik et al. 2014) have been shown to influence sperm competition.

Although contributions from the female reproductive tract are likely important in sperm competition, there are probably other contributions from the female outside of her reproductive tract. To examine the genes that contribute to sperm competition, without bias as to site of expression, an unbiased GWAS for female genes that influence variation in sperm competition was conducted (Chow et al. 2013). Briefly, two males from standard laboratory lines (*cn bw* [first male] and bw^D [second male]) were mated in sequence to females from 39 lines from the Drosophila Genetic Reference Panel (DGRP), which is a set of wild- derived, inbred lines whose

genome sequences are available (Mackay et al. 2012). The double mating experiments showed that sperm competition parameter P1 (proportion of first male progeny) was highly variable between DGRP lines. Statistical tests revealed single nucleotide polymorphisms (SNPs) that were associated with variation in the female's effect on sperm competition across DGRP lines. All SNPs are synonymous substitutions or are located in non-coding regions, suggesting that these SNPs somehow affect expression of candidate genes. Interestingly, 15 of the 33 top candidate genes identified by the GWAS are expressed in the nervous system or have specific neuronal functions. The functions of these neurological genes include encoding structural components of ion channels, and other aspects of nervous system function or development (Chow et al. 2013). The potential importance of the nervous system suggests that the female may be playing a more active role than previously thought in sperm competition outcome.

The role of neural genes in female reproductive success has been suggested in previous studies as well. *D. melanogaster* females are able to actively regulate the release of stored sperm through the neuromodulators octopamine (OA) and tyramine (TA; Avila et al. 2012). Both of these neuromodulators are synthesized in neurons that innervate the female reproductive tract (Middleton et al. 2006). While sperm entry and accumulation into storage were unaffected by the absence of OA and TA, sperm release from storage requires OA and TA (Avila et al. 2012). Furthermore, the uptake and release of several neuromodulators changes throughout the female reproductive tract after mating (Heifetz et al. 2014). This creates unique combinations of neuromodulator levels in different regions of the reproductive tract at different times, which may coordinate female post-mating responses such as sperm and egg movement and release, potentially influencing sperm competition outcomes.

The nervous system input has been shown to be important for sperm storage in other organisms as well. Neural input and hormones are important for muscular contraction in the spermatheca in locusts (for review, see Lange & da Silva 2007). More specifically, OA has been shown to modulate muscle contractions in the spermatheca in locusts (Clark & Lange 2003). Taken together, it appears that input from the female nervous system is important for proper sperm storage and may play a role in sperm competition. However how the nervous system works to control sperm use is unclear.

To examine the female's role in sperm competition, we first performed ubiquitous RNAi knockdowns of candidate genes from Chow et al. 2013 to assess the impact of decreased expression of these female-expressed candidate genes on sperm competition. Knockdown and control females were scored for sperm competition effects using progeny-phenotype assays. Of 29 genes tested, knockdown of 10 affected sperm competition outcomes. Effects on sperm competition outcome of knockdown of these genes in specific neurons were tested to identify those neurons that were essential for sperm competition. Separately, we examined the storage of sperm from multiple males after mating to female flies lacking octopamine or tyramine. Preliminary results suggest that octopamine and tyramine also play a role in how sperm is stored from multiple males over time. Overall, our results provide clues as to how females may actively influence sperm competition.

MATERIALS AND METHODS

Generating control and experimental females

To knock down female-expressed candidate genes, we generated RNAi females by crossing the ubiquitous driver *Tubulin*-GAL4/TM3, *Sb* virgin females to UAS-RNAi generating males (Vienna *Drosophila* RNAi Center [VDRC]). VDRC lines used are included in Table 1. Control females were generated by crossing virgin females from the same *Tubulin*-GAL4/TM3, *Sb* driver line to males from the *AttP* or w^{1118} RNAi background line (VDRC). Control and knockdown females were therefore identical except for the insertion of the RNAi transgene in experimental females. Only Sb^{+} progeny were used from each cross. Eighty to 100 virgin control and knockdown females were collected for each treatment. Typical sample size at the end was 30-60 doubly mated females, as females that did not mate the first or second time were not included in the final analysis. Knockdown efficiency (Table 1) was examined by PCR of cDNA synthesized from RNA isolated from whole control and knockdown female flies. A representative figure showing efficient knockdown is shown in Figure 1. In cases where knockdown was not determined, PCR was unsuccessful or knockdown has not yet been assessed.

In cases where ubiquitous knockdown of a candidate gene was lethal, a *Tubulin*-GAL80^{ts}/*Tubulin*-GAL80^{ts}*;* *Tubulin*-GAL4/TM3, *Sb* was used to allow for temporal control of GAL4 expression during adulthood only, by shifting crosses from room temperature to 29°C after flies had eclosed. Females were aged and maintained at 29°C throughout the assay.

We also examined candidates for a neural effect on sperm competition by driving knockdown using a pan-neuronal driver, *nSyb*-GAL4 (Hindle et al. 2013). If an effect was observed from pan-neuronal knockdown, we then used *ppk*-GAL4 (Matthews et al. 2007) to drive knockdown in the ppk neurons that innervate the female reproductive tract, and in one

case, *tdc2*-GAL4 (Cole et al. 2005), which targets octopaminergic neurons, which are important for sperm release from storage (Avila et al. 2012). All flies were collected as virgins under CO_2 anesthesia and aged 3-7 days in single-sex vials of 30-40 flies, and maintained on a yeast-glucose agar medium at room temperature on a 12-hour light/dark cycle.

Table 1. VDRC RNAi lines used for sperm competition assays.
N.D., Not Determined
% Knockdown, approximate percentage of gene knocked down

Gene	VDRC ID	% Knockdown
Cyp313a2	110504	N.D.
Shab	102218	90%
sima	106504	99%
spz5	102389	N.D.
CG42796	102356	N.D.
btsz	102608	90%
Msp300	107183	N.D.
CG10962	106396	90%
CG32532	106534	90%
para	104775	N.D.
SK	103985	N.D.
Rab2	105358	N.D.
Rim	39384	N.D.
hid	8269	N.D.
uif	101153	99%
CG9850	107199	99%
CG15800	110049	50%
CG32834	100964	80%
Rbp6	29799	99%
caup	105705	N.D.
CG32264	101503	80%
CG15765	101194	40%
CG33095	38604	90%
CG33298	42776	0%
CG31872	102669	99%
CG6163	22267	95%
Ddr	101831	99%
Zasp66	102980	97%
CG13594	36590	N.D.
Dh44	108473	N.D.

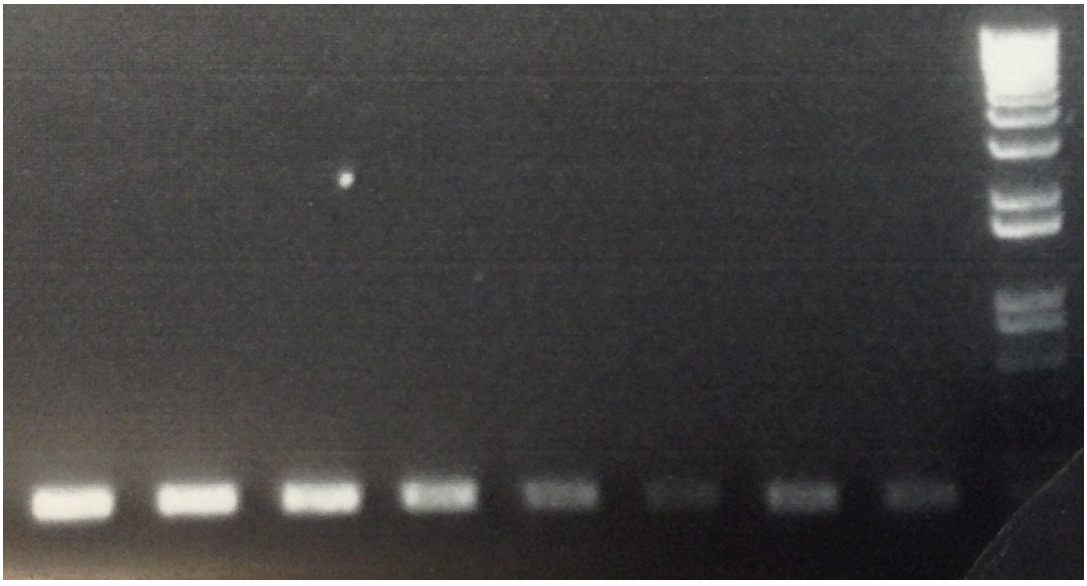


Figure 1. PCR showing knockdown efficiency of Zasp66 RNAi. The band is a 180 bp fragment of the cDNA of Zasp66. Control and knockdown samples were diluted as shown to gauge knockdown efficiency. Figure shows Zasp66 is knocked down about 97% (undiluted knockdown is comparable to control 1:40 dilution).

Sperm competition assays

Control and experimental females were given the opportunity to mate in sequence to males from the standard laboratory lines *cn bw* and bw^D. Sperm competition assays were performed by methods similar to those in Chow et al. (2013). Control and knockdown females have wild-type red eyes, while *cn bw* males have recessive mutations resulting in white eyes. The F_1 progeny sired by *cn bw* males have red eyes. The bw^D males are homozygous for a dominant mutation that causes brown eyes. F_1 progeny sired by bw^D males inherit one copy of the dominant bw^D allele, resulting in brown eyes. Young males were also collected at the same time as control and experimental females, and were handled and housed in a similar manner to virgin females.

For the first mating, virgin control and knockdown females were singly mated to *cn bw* males in glass vials (day 0; vial 1). Vials were observed for copulation every 15 minutes, and males were removed from vials after mating concluded. The next night (day 1), two bw^D males were placed with each female for the second mating for 12 hours overnight. In the morning on day 2, bw^D males were discarded and females were placed into a new vial (vial 2) and allowed to lay eggs for 48 hours. Again on days 4, 6, and 8, individual females were placed into a new vial (vials 3, 4, and 5) and allowed to lay eggs for 48 hours in each vial. On day 10, females were discarded. Adult female progeny were then scored for eye color (red vs. brown) to determine paternity. To be scored accurately, the bw^D eye color phenotype requires a w^+ background. Since VDRC lines as well as driver lines are in a w^- background, only female progeny are scored as they receive a w^+ chromosome from their bw^D fathers.

Statistical analysis

A P1 score was calculated for control and experimental females. P1 is the proportion of progeny sired by the first male after the second mating. In this case, this is represented by the number of red-eyed progeny divided by the total number of progeny (red/[red+brown]) from vials 2-5 of the individual females that remated. Since the second mating occurs in vial 1, vial 1 is not included in calculating the P1 score because it is not possible to determine which red-eyed progeny were deposited as embryos before the second mating occurred. If P1=0 or 1, we confirmed that there were both types of progeny in vial 1 (verifying that both matings occurred). P1 was then compared between control and experimental females using a one-way ANOVA. All statistical analyses were performed in R.

Examining sperm storage and dynamics from two different males in OA and TA mutant females

Experiments were carried out by doubly mating 3-5 day old virgin control or mutant females to 3-5 day old males that express a protamine labeled with either GFP (first male) or RFP (second male), which show green or red fluorescence, respectively, in sperm heads (Manier et al. 2010). Fly lines from which virgin control and mutant females were derived from were available in our lab ($tβh^{M18}$/FM7 and $tdc2^{RO54}$/*Gla*, Rubinstein and Wolfner 2013) and from the Bloomington Stock Center (*Df(2R)42/SM5*). All flies were maintained on a standard yeast-glucose medium at room temperature on a 12-hour light/dark cycle.

Double-mating experiments were done in a similar manner to the sperm competition assays described above. Virgin control ($tβh^{M18}$/*FM7*, $tdc2^{RO54}$/*SM5, Df(2R)42/Gla*) and virgin null-mutant ($tβh^{M18}/tβh^{M18}$, $tdc2^{RO54}$/*Df(2R)42*) females were first singly mated to GFP-sperm labeled males. GFP-sperm males were removed after mating was complete. The next day, all females who successfully mated with GFP-sperm labeled males were given the opportunity to

mate with two RFP-sperm labeled males overnight. The next morning the RFP-sperm male flies were removed, and females were maintained in vials for 1, 4, or 10 days, then flash frozen, and stored at -80°F to later be dissected. To visualize sperm, female reproductive tracts were dissected in PBS on a microscopic slide, covered with a class coverslip sealed with rubber cement, and examined at 400x under a florescence microscope.

The number of sperm stored within the individual sperm storage organs was counted for each time point at 400X on a fluorescence microscope. T-tests were used to determine if there was a difference in sperm stored from each type of male in each sperm storage organ over time.

RESULTS

Functional testing of candidate genes

To examine the possible role of candidate genes in sperm competition in females, we used RNAi to ask if reduced expression changes sperm competition outcomes. Of 29 genes tested, knockdown of 10 significantly affected P1 score (Table 2). Four candidates from Chow et al. (*CG34027, CG10858, RFeSP* and *sti*) do not have RNAi lines available, and one (*CG13594*) was not tested due to its 94 predicted off-targets. One additional candidate (*Dh44*) was tested due to its previously known involvement in sperm ejection (Kim et al. 2015). In cases where there is no ubiquitous knockdown shown, ubiquitous knockdown was too detrimental to females for a sperm competition assay to be performed (females did not survive through the assay). Additional tests were performed on some candidates to determine if using different types of males (Protamine-GFP and Protamine-RFP) and/or reversing the mating order of males would still show an effect on sperm competition outcomes.

Many candidates remained significant in their effect on P1 score when knocked down specifically in the nervous system as well. The neuronal candidates may be involved in the development of neural networks that are important for sperm competition, and/or could function at the time sperm competition occurs. Additional tests will need to be done to determine how candidates are acting to affect sperm competition outcomes.

Table 2. Measures of first male precedence (P1 score) in RNAi knockdowns of candidate genes. Ten of 29 genes tested affect P1 score when knocked down in females. P-values reflect the result of an ANOVA statistical test. Note: Knockdown of CG10962 results in a close-to-significant p-value. However, this RNAi line has 2 off targets and knockdown flies were not healthy.
KD, knockdown
N, number of doubly mated females
Median, median P1 score
Progeny, total progeny counted (missing in cases where experiment is from Chow et al. 2013)
*Significant result
† Data in row from Chow et al. 2013
‡ GFP- (first male) and RFP- (second male) sperm labeled males used for assay
§ Reversed mating order: bw^D first male, *cn bw* second male
‖ Candidate gene not from Chow et al. 2013 DGRP screen
Δ Data collected with Martik Chatterjee

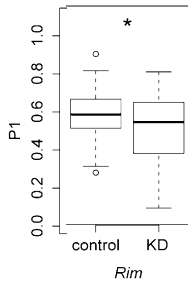
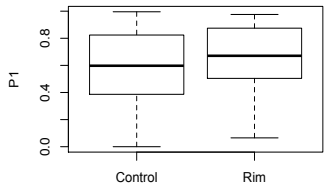
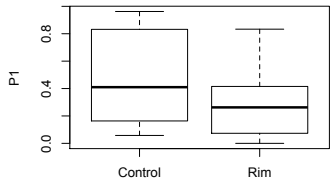
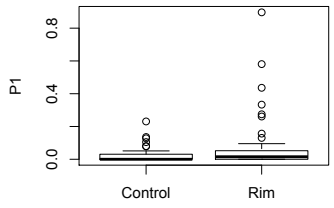
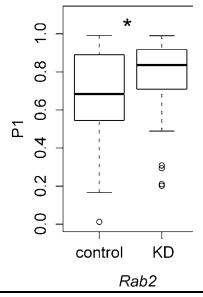
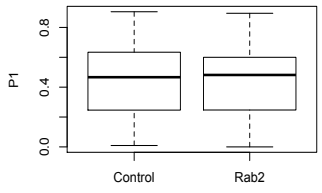
Gene knocked down	Driver	Control N, Median Progeny counted	KD N, Median Progeny counted	p-value	Box plot
Rim †	*ppk*-GAL4	44 0.560	41 0.519	0.017*	
‡	*ppk*-GAL4	64 0.597 16,304	51 0.671 13,762	0.2285	
	ppk-GAL4	32 0.41 3,881	34 0.263 4,105	0.0084*	
§	*ppk*-GAL4	42 0 5,053	57 0.015 8,060	0.0321*	
Rab2 †	*ppk*-GAL4	38 0.685	40 0.836	0.04*	
‡	*ppk*-GAL4	57 0.467 13,434	51 0.482 11,797	0.9605	

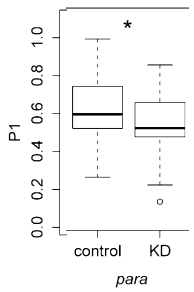
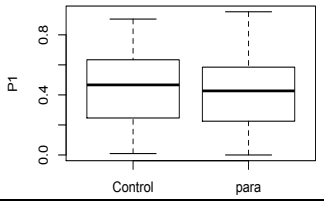
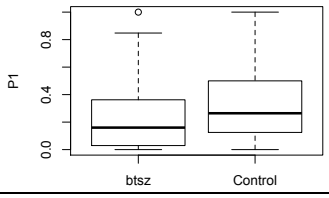
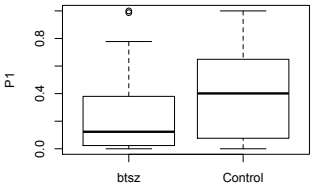
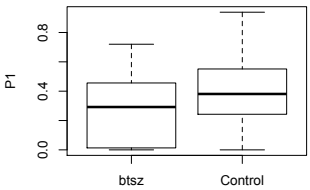
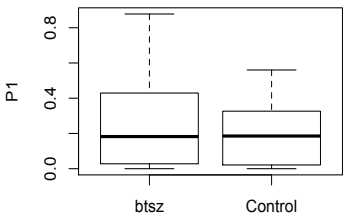
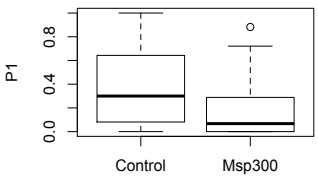
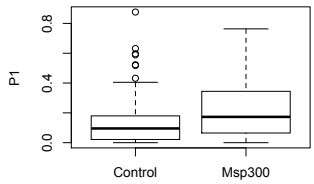
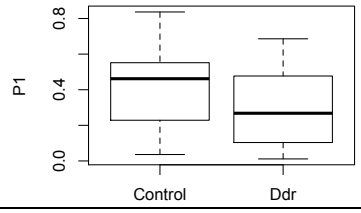
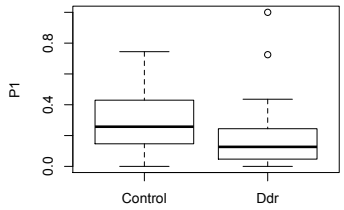
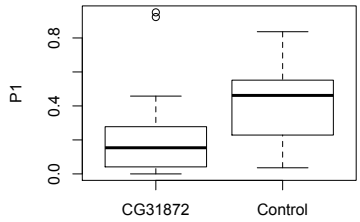
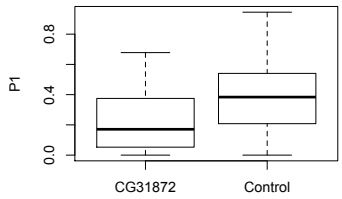
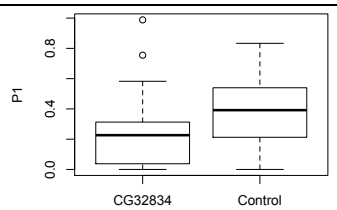
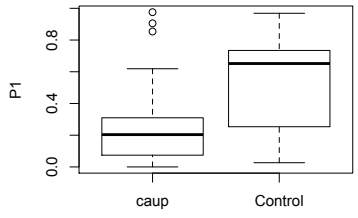
Table 2 (continued)					
para †	*ppk*-GAL4	40 0.58	42 0.506	0.008*	
‡	*ppk*-GAL4	57 0.467 13,434	35 0.427 8,335	0.5885	
btsz	*Tubulin*-GAL80^{ts}; *Tubulin*-GAL4/TM3,*Sb*	37 0.264 4,702	66 0.16 6,084	0.0281*	
Δ	*nSyb*-GAL4	46 0.402 6,538	52 0.123 6,284	0.003*	
	tdc2-GAL4	25 0.381 3,404	14 0.292 1,738	0.1386	
Δ	*ppk*-GAL4	34 0.185 4,927	40 0.182 5,603	0.2637	
Msp300	*Tubulin*-GAL80^{ts}; *Tubulin*-GAL4/TM3,*Sb*	85 0.3 7,812	48 0.067 2,735	0.0002*	

Table 2 (continued)					
	nSyb-GAL4	66 0.095 8,010	58 0.173 7,538	0.04*	
Ddr	*Tubulin*-GAL4/TM3,*Sb*	38 0.462 5,404	25 0.268 2,479	0.015*	
	nSyb-GAL4	34 0.257 4,914	36 0.127 3,969	0.0311*	
CG31872	*Tubulin*-GAL4/TM3,*Sb*	38 0.462 5,404	33 0.154 3,872	0.0001*	
	Tubulin-GAL4/TM3,*Sb*	61 0.384 7,828	22 0.171 1,777	0.0073*	
CG32834	*Tubulin*-GAL4/TM3,*Sb*	36 0.392 4,101	43 0.226 4,316	0.0038*	
caup Δ	*nSyb*-GAL4	24 0.652 2,447	33 0.204 3,726	0.0015*	

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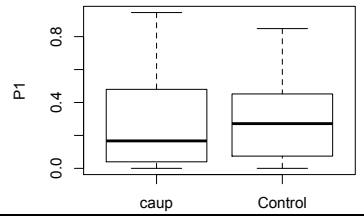
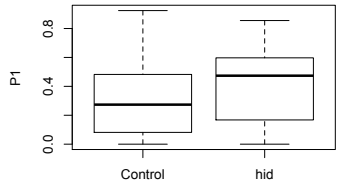
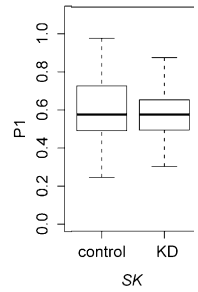
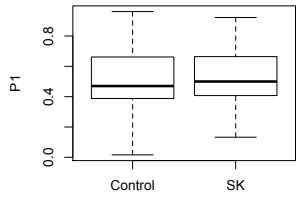
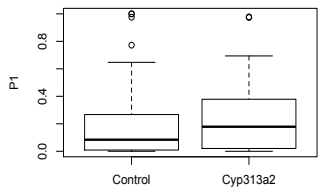
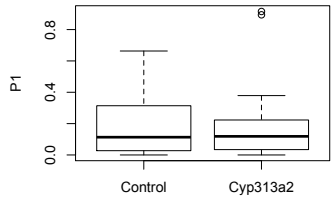
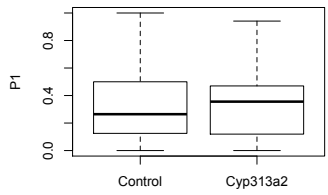
	ppk-GAL4	55 0.272 5,748	55 0.167 5,722	0.5753	
hid	*nSyb*-GAL4	44 0.273 10,096	57 0.474 12,257	0.0170*	
SK †	*ppk*-GAL4	40 0.578	40 0.578	0.344	
Δ	*nSyb*-GAL4	49 0.47 6,737	52 0.5 7,216	0.9797	
Cyp313a2	*Tubulin*-GAL80^{ts}; *Tubulin*-GAL4/TM3,*Sb*	62 0.084 8,569	58 0.179 6,904	0.1916	
	Tubulin-GAL80^{ts}; *Tubulin*-GAL4/TM3,*Sb*	46 0.113 6,350	41 0.119 5,742	0.8458	
	Tubulin-GAL80^{ts}; *Tubulin*-GAL4/TM3,*Sb*	37 0.264 4,702	48 0.356 5,824	0.9591	

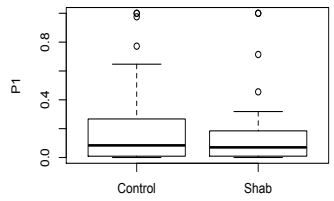
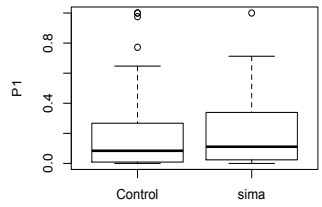
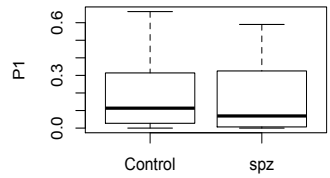
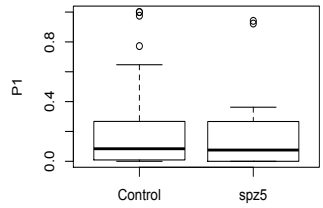
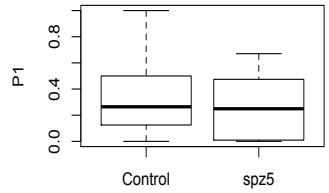
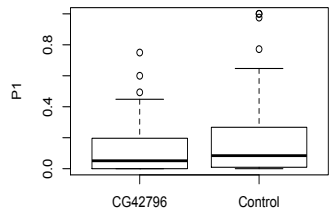
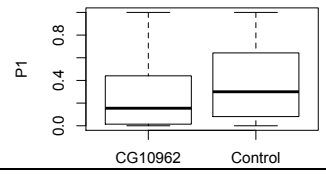
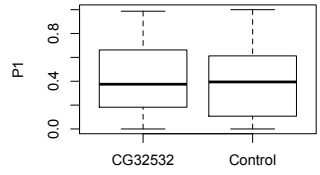
Table 2 (continued)					
Shab	*Tubulin*-GAL80^{ts}; *Tubulin*-GAL4/TM3,*Sb*	62 0.084 8,569	66 0.07 7,290	0.157	
sima	*Tubulin*-GAL80^{ts}; *Tubulin*-GAL4/TM3,*Sb*	62 0.084 8,569	36 0.111 3,882	0.5363	
spz5	*Tubulin*-GAL80^{ts}; *Tubulin*-GAL4/TM3,*Sb*	46 0.113 6,350	16 0.069 1,190	0.9049	
	Tubulin-GAL80^{ts}; *Tubulin*-GAL4/TM3,*Sb*	62 0.084 8,569	41 0.075 4,894	0.4446	
	Tubulin-GAL80^{ts}; *Tubulin*-GAL4/TM3,*Sb*	37 0.264 4,702	16 0.249 1,981	0.325	
CG42796	*Tubulin*-GAL80^{ts}; *Tubulin*-GAL4/TM3,*Sb*	62 0.084 8,569	40 0.051 2,856	0.2161	
CG10962	*Tubulin*-GAL80^{ts}; *Tubulin*-GAL4/TM3,*Sb*	85 0.3 7,812	36 0.155 2,217	0.0707	
CG32532	*Tubulin*-GAL4/TM3,*Sb*	45 0.394 6,486	31 0.375 2,936	0.6465	

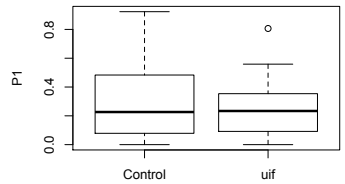
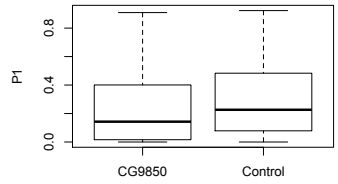
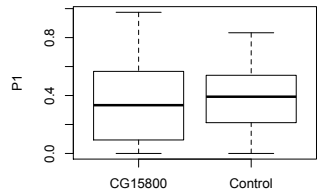
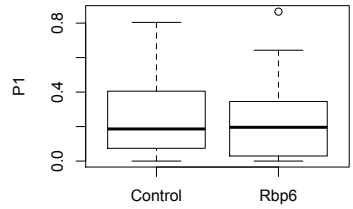
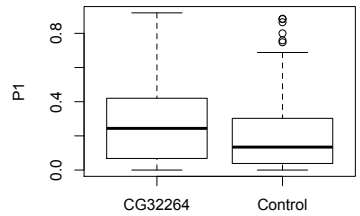
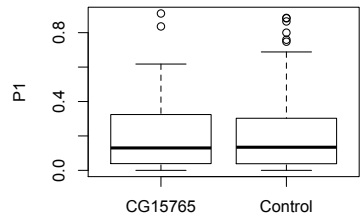
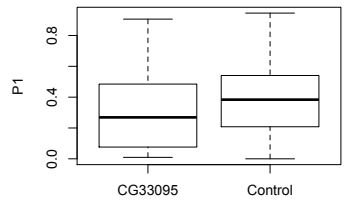
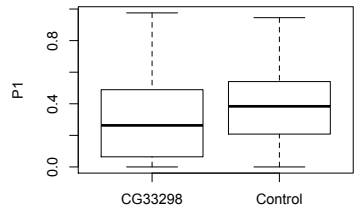
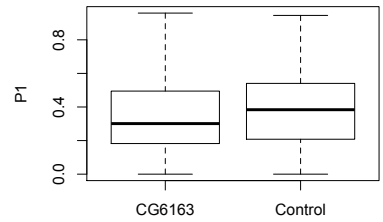
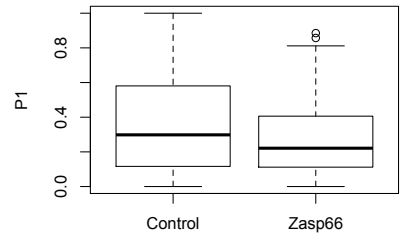
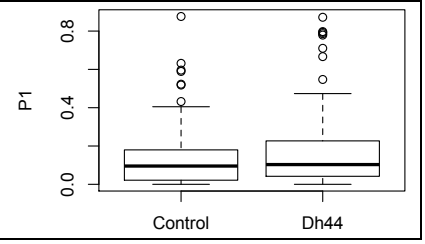
Table 2 (continued)					
uif	*Tubulin*-GAL80^{ts}; *Tubulin*-GAL4/TM3,*Sb*	44 0.226 6,255	30 0.234 2,927	0.5673	
CG9850	*Tubulin*-GAL80^{ts}; *Tubulin*-GAL4/TM3,*Sb*	44 0.226 6,255	39 0.143 4,397	0.5681	
CG15800	*Tubulin*-GAL4/TM3,*Sb*	36 0.392 4,101	48 0.333 4,349	0.5083	
Rbp6	*Tubulin*-GAL80^{ts}; *Tubulin*-GAL4/TM3,*Sb*	41 0.186 4,913	27 0.196 2,780	0.8464	
CG32264	*nSyb*-GAL4	51 0.134 7,160	25 0.244 3,355	0.4775	
CG15765	*nSyb*-GAL4	51 0.134 7,160	40 0.13 5,534	0.7024	
CG33095	*Tubulin*-GAL4/TM3,*Sb*	61 0.384 7,828	27 0.269 3,014	0.2967	
CG32298	*Tubulin*-GAL4/TM3,*Sb*	61 0.384 7,828	41 0.263 4,452	0.3175	

Table 2 (continued)					
CG6163	*Tubulin*-GAL4/TM3,*Sb*	61 0.384 7,828	35 0.301 4,078	0.6127	
Zasp66	*Tubulin*-GAL4/TM3,*Sb*	29 0.298 2,402	60 0.221 6,209	0.0642	
Dh44 ‖	*nSyb*-GAL4	66 0.095 8,010	67 0.103 8,879	0.2965	

Octopamine and Tyramine may play a role in sperm dynamics

Octopamine and tyramine have been previously been shown to be important for sperm storage (Avila 2012). To examine whether they are also important for differential sperm storage/use from two different males, we used fly lines mutant in genes that encode enzymes that synthesize tyramine (*tyrosine decarboxylase 2* [*tdc2*], Cole et al. 2005) and octopamine (*tyramine β-hydroxylase* [*tβh*], Monastirioti et al. 1996). We mated females lacking octopamine (*tβh*), or tyramine and octopamine (*tdc2*) to males with green- or red-labeled sperm (Protamine-GFP [first male] and Protamine-RFP [second male], Manier et al. 2010), and examined sperm storage 1, 4, and 10 days after the second mating.

One day after the second mating, *tβh* females (lacking octopamine) have significantly more first male sperm in the seminal receptacle, less second male sperm in the seminal receptacle, and more total sperm compared to controls. By 4 days after the second mating, octopamine mutant females are overall maintaining more sperm in storage compared to controls. The most striking difference here is that controls have much less sperm remaining in the seminal receptacle at this time point. Ten days after the second mating, controls have almost no sperm remaining in the seminal receptacle, while mutants still retain first male's sperm. No second male's sperm is detected in mutants by 10 days after the second mating, so it appears that mutants are also releasing second male sperm faster than controls (Figure 2, Table 3). These results are similar for *tdc2* females (lacking tyramine and octopamine; Figure 3, Table 4).

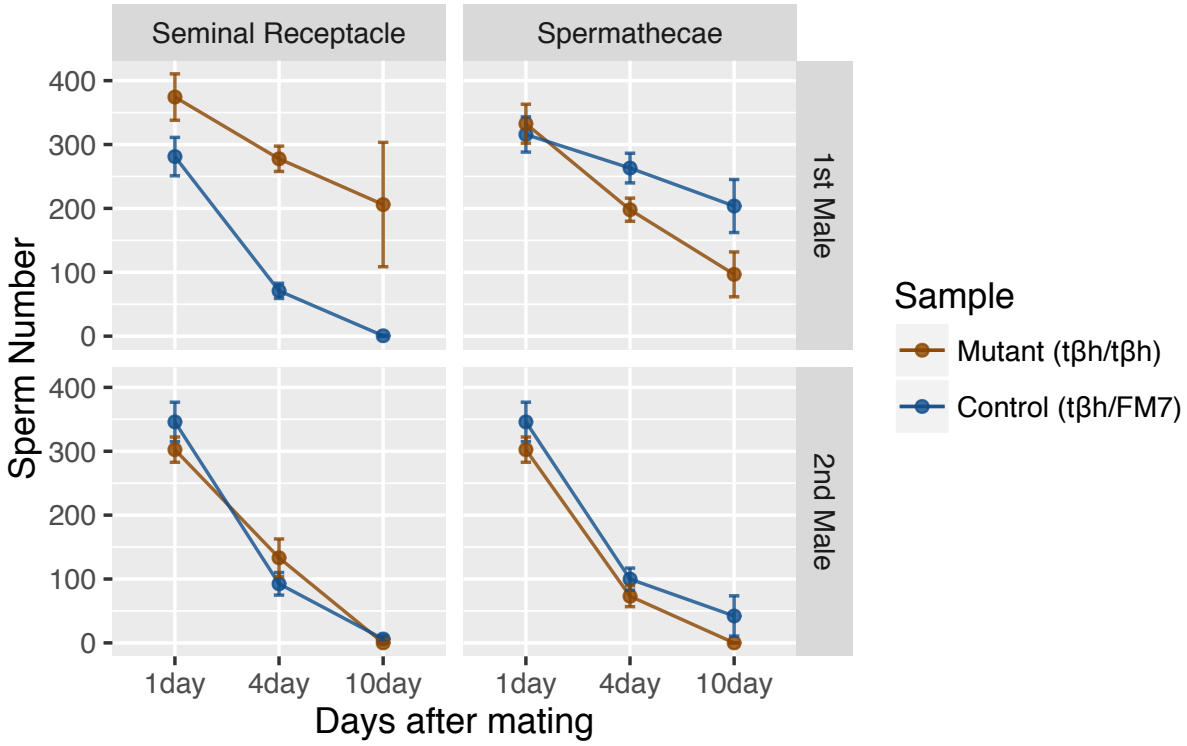


Figure 2. Control and *tβh* mutant females' sperm storage from 2 different males. ProtamineB-GFP is the first male, ProtamineB-RFP is the second male.

Table 3. Control and *tβh* mutant females' sperm storage from 2 different males. ProtamineB-GFP is the first male, ProtamineB-RFP is the second male. Means are represented in the table (variation can be observed in Figure 2).
N, number of females (Mutant, Control)
NS, not significant
*Significant result

		Seminal Receptacle			Spermathecae		
		1 day N (16,19)	4 day N (18,16)	10 day N (24,32)	1 day N (16,19)	4 day N (18,16)	10 day N (24,32)
1st male	Mutant	374.3	277.7	206	332.6	197.4	96.7
	Control	281.1	70.9	0.6	315.7	263.1	203.7
	p-value	4.9e-09*	2.2e-16*	4.1e-10*	0.0983	7.6e-10*	1.8e-14*
2nd male	Mutant	302.5	133.2	0	178.2	73.1	0
	Control	345.9	92.5	6.1	180.1	99.9	42.2
	p-value	1.9e-05*	3.1e-05*	3.6e-08*	0.806	5.9e-05*	1.6e-08*

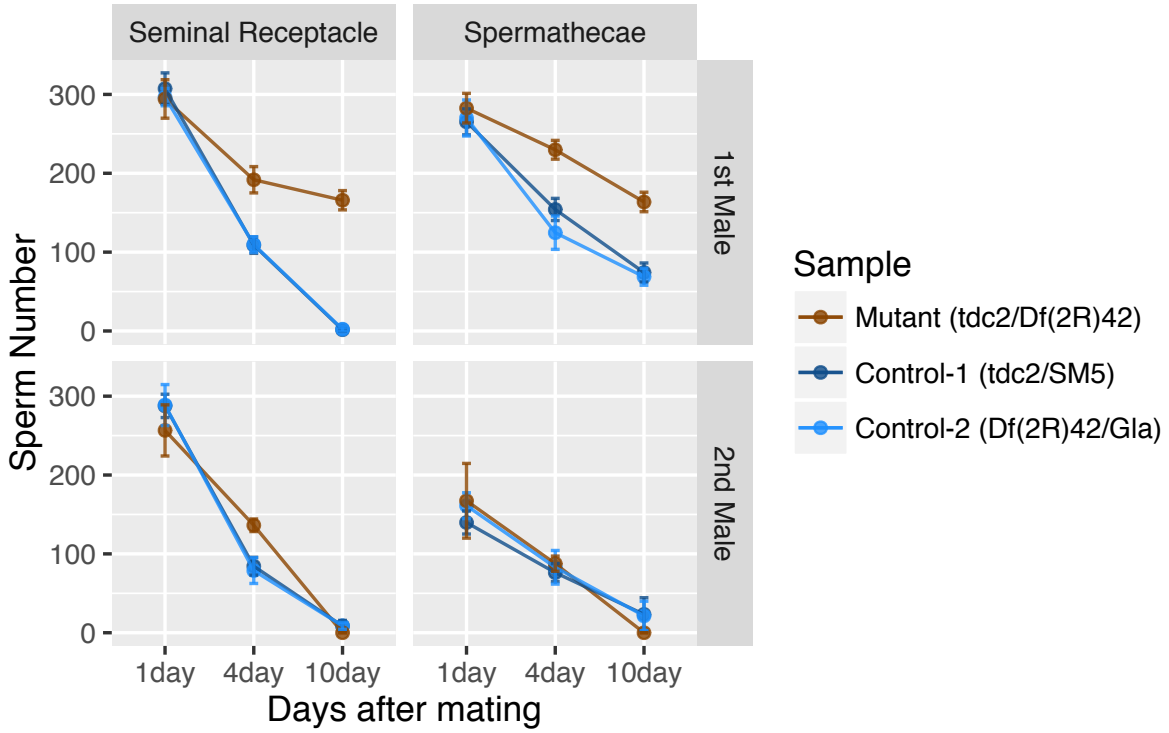


Figure 3. Control and *tdc2* mutant females' sperm storage from 2 different males. ProtamineB-GFP is the first male, ProtamineB-RFP is the second male.

Table 4. Control and *tdc2* mutant females' sperm storage from 2 different males. ProtamineB-GFP is the first male, ProtamineB-RFP is the second male. Means are represented in the table (variation can be observed in Figure 3).
N, number of females (Mutant, Control-1, Control-2)
NS, not significant
*Significant result

		Seminal Receptacle			Spermathecae		
		1 day N(12,9,7)	4 day N(14,8,9)	10 day N(16,7,11)	1 day N(12,9,7)	4 day N(14,8,9)	10 day N(16,7,11)
1st male	Mutant	294.3	191.8	165.8	282.6	229.8	163.6
	Control-1	307.3	108.9	1.6	265.2	154.1	74.1
	Control-2	296.6	109.2	2.3	270.1	124.7	68.7
	p-value (Control-1, Control-2)	0.1935 0.7801	6.7e-12* 1.4e-12*	2.2e-16* 2.2e-16*	0.0366* 0.2485	1.2e-08* 2.3e-08*	2.1e-09* 2.2e-16*
2nd male	Mutant	256.6	136.3	0	167.3	87.6	0
	Control-1	287.7	84.4	8.6	139.8	76.3	23.3
	Control-2	288.7	78.8	8.7	161.1	83	21.4
	p-value (Control-1, Control-2)	0.0096* 0.0312*	1.8e-07* 1.2e-06*	0.0186* 5.8e-05*	0.0791 0.686	0.0339* 0.5586	0.0268* 0.0035*

DISCUSSION

Although there is copious evidence that female genotype is important for sperm competition outcome (Clark and Begun 1998; Clark et al. 1999; Chow et al. 2010), very little is known about the specific genes and mechanisms involved in the female contribution. Here, we show that 10 of 29 candidate genes when knocked down in females affect P1 score, the proportion of offspring sired by the first male in doubly mated females.

Many complex interactions occur between males and females, and the genes we identified as involved in sperm competition outcome could be acting at any point in time before, during, or after mating. For example, a candidate gene may be involved in pre-copulatory interactions, such as detecting males' courtship behaviors or pheromones. A significant effect on P1 score could also arise from variation in remating behavior, copulation duration, or other nervous system controlled functions such as allocation of sperm into storage, their retention there, or their release from storage. Further tests should be conducted with these candidate genes to determine through which neurons they are acting and if they may have an effect on pre-copulatory behaviors.

Here, we also show that the neuromodulators octopamine and tyramine may play a role in sperm dynamics as there are significant differences in how mutant and control females store sperm from two different types of males. This is a possible physiological mechanism by which females actively modulate sperm use from different males. In the future, it would be interesting to test if the 10 candidate genes we confirmed are important in octopaminergic and/or tyraminergic neurons for sperm competition outcome.

Both of these sets of experiments suggest that one role of the female nervous system is to bias sperm use, suggesting a more active role in sperm competition by females than previously

thought. Future studies should focus on how these candidates are affecting sperm competition and through which neurons they are acting. Then, when we have a more comprehensive view of how males and females affect sperm competition outcome separately, we can begin to examine more complex interactions between males and females.

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