Supplementary material

Fluorescent marker donor vectors

*Nde*I restriction sites were introduced at the 5'- and 3'-ends of fluorescent markers eGFP, mTurquoise and mCherry by amplification with primers *Nde*GFP ('5-tgcatatgcaaggtgagcaagggcgaggagctgttcacc-3') and pKC26fB2,0.Fp-rev (5'-gcccatatgttacttgtacagctcgtccatgc-3') for eGFP and mTurquoise and primers *Nde*mCherry (5'-tgcatatgcaaggtgagcaagggcgcggaggataacat-3') and pKC26fB2,0.Fp-rev for mCherry. Fragments were ligated into pJET1.2 vector (Thermo Scientific) by blunt-end ligation and cloned XL1-blue colonies were screened by restriction analyses.

Protamine B::fluorescent marker CDS

In order to create a CDS for Protamine B fusion protein with integrated eGFP, mTurquoise of mCherry, fluorescent markers were excised from the subcloned pJET1.2 plasmids using *Nde*I and ligated into the pBS/ProtB4.2KP*Nde*I (Manier et al., 2010) vector. Fragments were integrated in frame with the CDS on position 45 of exon 3 of the ProtB ORF via an introduced *Nde*I restriction site. Orientation of the fluorescent fragments (3' to 5') in the pBS/ProtB4.2KP-NdeI vector were determined by restriction analysis using *Dpn*I.

Fluorescent protamine B cloning and expression vector

To create vectors for generating *Drosophila* expressing Protamine B::fluorescent marker fusion proteins, 4.8Kb *Xba*I fragments of the different pBS/ProtB4.2KP constructs containing the introduced florescent markers were subcloned into the *Xba*I side of plasmid pUAST-attB1. The vector used in this study contains an inactivated 5x UAS-hsp70 site, obtained after digestion with *Pst*I and religation of the 8.2Kb fragment. Orientation of the

inserted protamineB::fluorescent marker CDS was determined by restriction analysis with BamHI. Sequence analysis of the reading frame of fluorescent markers and conformation of the direction of the protamineB::fluorescent marker CDS in pUAST-attB-ProtB::eGFP and pUAST-attB-ProtB::mTurquoise was performed using the primers Seq-ProtB-for (5'-ggacctgtcactaacaac-3'), Seq-GFCITU-rev (5'-gatgttgtggcggatctt-3') and SV40poly(A).1-rev (5'caccacagaagtaaggttcct) and primers Seq-ProtB-for, Seq-ProtB-rev (5'-gcgctattccaacatccta-3') and SV40poly(A).1-rev for pUAST-attB-ProtB::mCherry.

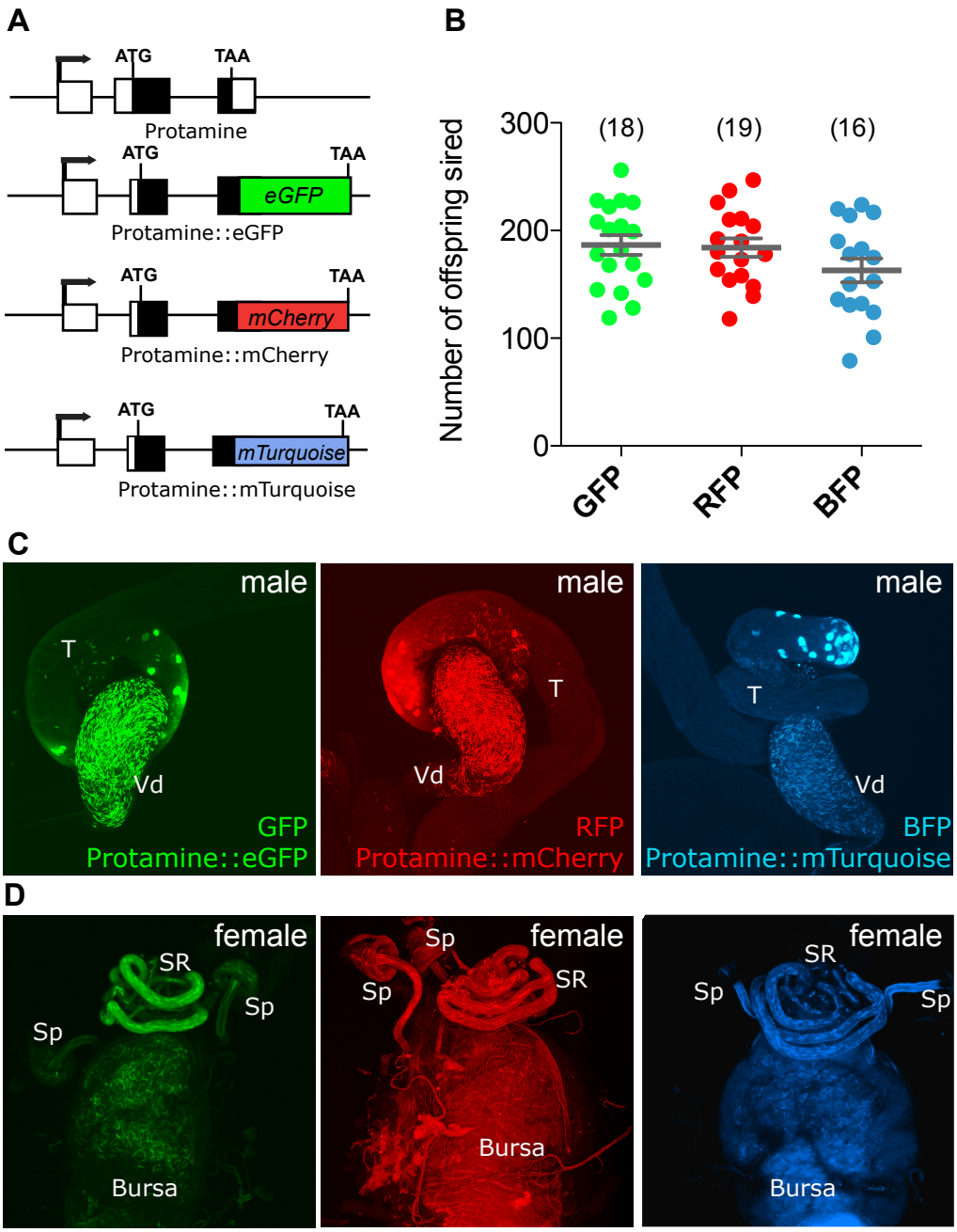
Generation of transgenic Drosophila

To generate transgenic *Drosophila* with eGFP, mTurquoise or mCherry expressing sperm heads, the different pUAST-attB vectors with protamine B::fluorescent marker CDS were used to inject PhiC31-containing attP docking site embryos of *Drosophila melanogaster* with genotype y[1] M{vas-int.Dm}ZH-2A w[*]; M{3xP3-RFP.attP}ZH-102D (BDSC stock number 24488). Embryo injection and generation of stable transformants was performed at BestGene Inc., U.S.A.

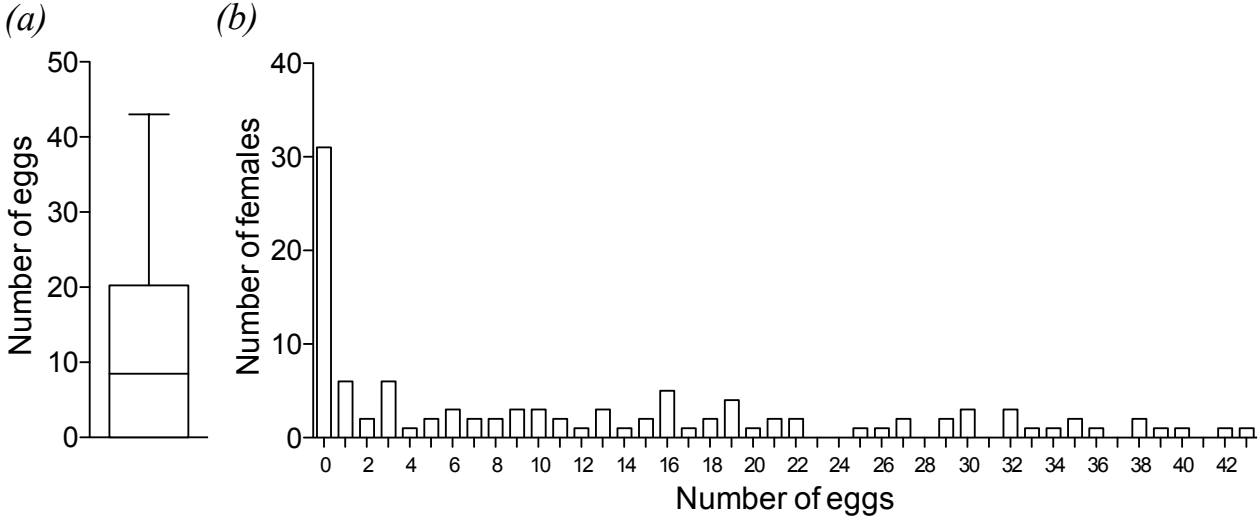
	1st mating				Interval		2nd mating				Offspring	
	males	female	Dur	Var	Isolated	Dur	males	female	Dur	Proof	Genotyping	Dur
Boorman and Parker 1976	1	1	NS	Ob	yes	4-14 days	1	1	5	Ob	irradiation treatment	life-time
Lefevre and Jonnson 1962	1	1	NS	Ob	no	0-24 hrs	1	1	ns	Ob/Off	mutant, forked	life-time
Hughs 1997a	1	2	2	Off	yes	3 days	1	2	24	Off	mutant, eye colour	10
Hughs 1997b	en masse	en masse	NS	Off	yes	3 days	1	2	24	Off	mutant, eye colour	10
Clark et al. 1995	en masse	en masse	2	Off	yes	2,5 days	1	3	12	Off	mutant, eye colour	13
Prout and Bundgaard 1977	100	100	2	off	yes	36	1	3	24	Off	mutant, eye colour	12
Clark et al. 1998	en masse	en masse	2	Off	yes	2 days	1	2 or 3	18	Off	mutant, eye colour	13
Gilbert and Richmond 1981a	1	1	NS	Ob	yes	daily	1	1	2	Ob	mutant, forked	7
Gilbert and Richmond 1981b	varied	varied	7 days	Off	no	none	NA	NA	NA	NA	mutant, forked	9
Gilbert and Richmond 1981c	1	1	24	Off	no	0	1	1	24	Off	mutant, forked/eye-colour	8
Morrow et al. 2005	16	24	1,5	Off	no	(0-24)	16	16	24	Off	mutant	<24 hrs
Chapman et al. 2000a	6	7 to 9	NS	Ob	yes	2 day	2	1	18	Off	mutant, eye colour	13
Chapman et al. 2000b	1	1	NS	Ob	yes	3-5 days	1	1	NS	Ob	irradiation treatment	lifetime
Chapman et al. 2000c	6	7 to 9	NS	Ob	yes	1-2 day	2	1	18	Off	mutant, eye colour	13
Scott and Richmond 1990	1 or 2	1	NS	Ob	yes	6 hrs	1	1	1	Ob	yellow body, attached X	6
Harshman and Prout 1994a	20	20	24	Off	yes	4 days	20	20	48	Off	mutant, eye colour	20
Harshman and Prout 1994b	20	20	24	Off	yes	4-6 days	2	1	3	Ob	used sterile males	20
Price et al. 1999	1	1	10	Ob	yes	1 day	1	1	10	Ob	mutant, eye colour	life-time
Price 1997	1	1	10	Ob	yes	1 day	1	1	10	Ob	mutant, eye colour	life-time
Civetta and Finn 2014	1	1	8	Ob	yes	2 days	1	1	8	Ob	mutant, body colour	life-time
Manier et al. 2010	1	1	NS	Ob	yes	3 days	1	1	6	Ob	fluorescent protein	varied
Clark and Begun 1998	en masse	en masse	2	Off	yes	2 days	2 or 3	1	18	Off	mutant, eye colour	11
Civetta and Clark 2000	en masse	en masse	2	Off	yes	2,5 days	1	3	12	Off	mutant, eye colour	13
Clark et al 1999	en masse	en masse	2	Off	yes	2 days	2 or 3	1	18	Off	mutant, eye colour	11
Ala-Honkola et al. 2014	NS	NS	NS	Off	NS	2-5 days	1	1	4	Ob	fluorescent protein	6

Billeter et al. 2012	6	6	8	Ob	no	none	6	6	8	Ob	cuticular pigment pattern	life-time
Bjork et al. 2007	1	1	NS	Ob	yes	3-4 days	1	1	NS	Ob	mutant, eye colour	11
Amitin and Pitnick 2007	1	1	NS	Ob	yes	2-8 days	1	1	5	Ob	mutant, eye colour	1
Mack et al. 2003	40	65	3	Off	yes	3 days	2	1	18	Off	mutant, eye colour	12
Bangham et al. 2003	1	1	6	Ob	yes	3 days	1	1	9	Ob	mutant, eye colour	life-time
Reinhart et al. 2015	1	1	6	Ob	yes	3 days	2	1	6 or 12	Ob/Off	mutant, eye colour	2 or 3

Supplementary Table S1. Survey of the experimental design used to investigate last male sperm precedence. Publications listed with a lower case letter indicate multiple experiments within the same report. Dur = duration, Var = method of verification of mating, Ob = observed, Off= offspring



Supplementary Figure 1. Transgenic males expressing fluorescent protein labeled sperm heads. ***(a)*** Representation of genetic constructs derived from the ProtamineB gene. Arrow indicates the start site of transcription; ATG and TAA the Open Reading frame; boxes indicate exons. ***(b)*** Mean number of offspring produced by once-mated females mated to one of the three transgenic males expressing fluorescent protein labeled sperm; green fluorescent protein (GFP), red fluorescent protein (RFP, specifically mCherry), blue fluorescent protein (BFP, specifically mTurquoise). Differences between groups were assessed with a One-way ANOVA ($F(2, 48) = 1.767$, $p = 0.18$). Error bars indicate s.e.m. Number of replicates is between brackets. ***(c)*** Micrographs of male testes expressing fluorescent protein labeled sperm heads: GFP, RFP, and BFP, respectively. Location of the testes (T) and vas deferens (Vd), are indicated. Images are maximum projections of confocal stacks. ***(d)*** Micrograph of female sperm storage organs and anterior uterus of wild-type female mated to one of the three transgenic males: GFP, RFP, and BFP, respectively. Location of seminal receptacle (SR), paired spermathecae (Sp); and uterus/bursa (Bursa) are indicated.



Supplementary figure 2. Number of eggs laid by females in the mating chamber. A) Box plot graph of the numbers of eggs laid during the assay. Box represents the interquartile range, the segment inside the box indicates the median, and the wisker above shows the maximum range. B) Histogram of the number of eggs laid by females in the mating chamber. n = 110.