

Loss-of-function in testis-specific serine/threonine protein kinase (TSSKs) triggers male infertility in an invasive moth

Zihan Wei^{1,2,3, a}, Yaqi Wang^{1,2, 3, a}, Kangwu Zheng^{1,2, 3}, Zhiping Wang^{1, 2, *},
Ronghua Liu^{1,2, 3}, Pengcheng Wang, Yuting^{1,2,3}, Li^{1, 2, 3}, Ping Gao^{1, 2, 3}, Xueqing Yang^{1, 2, 3, *}

¹ College of Plant Protection, Shenyang Agricultural University, Shenyang 110866, Liaoning, China.

² Key Laboratory of Economical and Applied Entomology of Liaoning Province, China

³ Key Laboratory of Major Agricultural Invasion Biological Monitoring and Control, Shenyang 110866, Liaoning, China

^a These authors contributed equally to this work.

* Correspondence to: X-Q Yang (sling233@hotmail.com), Orcid ID: 0000-0002-3919-8013;

Z-P Wang (wangzp@syau.edu.cn).

Table S1. Gene information utilized for phylogenetic analysis.

Gene symbol	GenBank
PxTSSK2	XP_037963254.1
SfTSSK1	XP_035431594.1
SfTSSK2	XP_035449534.1
CiTSSK1	XP_034949554.1
SlTSSK1	XP_022828857.1
SlTSSK2	XP_022817768.1
BmTSSK4	XP_021206330.1
PmTSSK1-1	KPJ12526.1
PmTSSK2	KPJ18182.1
PmTSSK1-2	XP_014361874.1
PmTSSK4	KPJ09623.1
OfTSSK2	XP_028165800.1
OfTSSK1	XP_028163804.1
MsTSSK4	XP_037295123.1
MsTSSK2	XP_030024693.1
MsTSSK1	XP_037297243.1
HaTSSK2	XP_021186460.1
HaTSSK1	XP_021183931.1
ZcTSSK1	XP_011192630.1
DsTSSK1	XP_016943921.1
CcTSSK1	XP_004536887.1
DaTSSK1	XP_001954102.1
LsTSSK1	XP_037806454.1
LcTSSK1	XP_023305836.2
LcTSSK4	KAI8121587.1
LcTSSK2	XP_046812453.1
DbTSSK2	XP_017835088.2
BoTSSK4	XP_014096334.1
DmTSSK4	XP_033163513.1
CcTSSK4	XP_012158274.1

Table S2. Primers used in this study.

Primer name	Sequence (5'-3')
TSSK1-sgRNA-F	GCGGATATGGACGCGGCAGCTGG
TSSK1a-sgRNA-F	GCGGCAAGTTACCCAGCTGATGG
TSSK2-sgRNA-F	GTACACAGTATTCTGCAGCGAGG
TSSK2a-sgRNA-F	GGACCAGGTAACGCAGTTGATGG
TSSK4-sgRNA-F	GGACTCTGATATATGGAGCATGG
sgRNA-R	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCC TTATTTTAACTTGCTATTTCTAGCTCTAAAAC
KO-TSSK1-F	ACCTCGCAGAGTACAAGAATCCT
KO-TSSK1-R	AATCCATCGCAATCCATTCACTA
KO-TSSK1a-F	CGCTGTGCCCCGAGAACCA
KO-TSSK1a-R	CTGTAGAGCATCACCGAACCCT
KO-TSSK2-F	AGCGCCTGAGCCCTCGAA
KO-TSSK2-R	CACAAGCCGCCCCACCAT
KO-TSSK2a-F	GCGTGCCTTGACTCGTATTTTC
KO-TSSK2a-R	GCAACCGACTTGATTGACCAC
KO-TSSK4-F	CGACATAATCCGTAAGGATCAGCA
KO-TSSK4-R	TACACGATAGCGTAAAGAACAACCC
T7-RNAi-TSSK1-F	GGATCCTAATACGACTCACTATAGGAGCAGCAAATGAGCAAGAAGT
T7-RNAi-TSSK1-R	GGATCCTAATACGACTCACTATAGGTGGATTGTTTAATATGTCACCGT
T7-RNAi-TSSK1a-F	GGATCCTAATACGACTCACTATAGGAGCTGCGGTCTCCACTACAT
T7-RNAi-TSSK1a-R	GGATCCTAATACGACTCACTATAGGGACCGTTAAAGAGGCTGCTG
T7-RNAi-TSSK2-F	GGATCCTAATACGACTCACTATAGGGTACCCGAAAACCAGGCTAAA
T7-RNAi-TSSK2-R	GGATCCTAATACGACTCACTATAGGCGTAAGTTGGAGTCATCAAAAAGG
T7-RNAi-TSSK2a-F	GGATCCTAATACGACTCACTATAGGATATGTGGTCCGTGGGTATT
T7-RNAi-TSSK2a-R	GGATCCTAATACGACTCACTATAGGCAAGCAGTTGTTTCATTGGTG
T7-RNAi-TSSK4-F	GGATCCTAATACGACTCACTATAGGAAATAATAAGCAAGTTCCAAGCG
T7-RNAi-TSSK4-R	GGATCCTAATACGACTCACTATAGGCAGGGCATAACCCCGTTC
qPCR-TSSK1-F	CGGATTTGTGGTCGCTGGGT
qPCR -TSSK1-R	GTTCAACACTTGACACGGCCG
qPCR -TSSK1a-F	GTAAAGTGATTGACACGACGGGAGC
qPCR -TSSK1a-R	ACGATGTGCGGATGGTTGAGGC
qPCR -TSSK2-F	TGATGCGTCCAGTCCCAAGC
qPCR -TSSK2-R	GGCCTCGCTGCAGAATACT
qPCR -TSSK2a-F	TCAACCACCCTCACATCATACC
qPCR -TSSK2a-R	CTGTCTCGTCCACAATCTGCTC
qPCR -TSSK4-F	TGGACTIONGCCACGAACGC
qPCR -TSSK4-R	TGCCGCAGAAGGTTTCACTCA

Table S3. Detection of Mutation Rate in the TSSKS Gene Using CRISPR/Cas9.

Treatments	Inject embryos	Hatching rate (%)	Mutation rate (%)
sgRNA-TSSK1	265	42.35%	10.07%
sgRNA-TSSK1a	211	35.89%	12.64%
sgRNA-TSSK2	295	31.63%	11.28%
sgRNA-TSSK2a	289	42.65%	9.38%
sgRNA-TSSK4	378	35.42%	11.48%
Cas9	255	39.34%	
WT	274	75.63%	

Table S4. Biological parameters of *Cydia pomonella* with eggs injected with Cas9 and sgRNA.

Treatments	Oviposition period -female (days)	Oviposition period -male (days)	Larval period -female (days)	Larval period -male (days)	pupal period -female (days)	pupal period -male (days)	adult period -female (days)	adult period -male (days)
Cas9+sgRNA	5.14±0.50 a	5.24±0.35 a	19.78±2.13 a	20.12±1.16 a	9.53±0.64 a	10.10±0.84 a	15.14±1.24 a	21.03±2.18 a
Wt	5.44±0.51 a	5.06±0.28 a	20.44±2.25 a	19.41±2.23 a	10.15±0.97 a	9.81±0.71 a	14.04±1.76 a	20.98±1.81 a
Cas9	5.16±0.48 a	5.35±0.27 a	20.11±3.14 a	20.26±1.78 a	10.23±0.79 a	9.13±0.89 a	15.16±2.14 a	21.13±2.69 a

Significant differences ($P < 0.05$) between treatment groups were identified through one-way analysis of variance (ANOVA).

Table S5. The prediction results of lnc117962-miRNA information using different tools.

miRNA	RNAhybrid	PITA	miRanda	Total
Cpom-miR-11975	1	1	0	2
Cpom-miR-1814	0	1	1	2
Cpom-miR-2861	1	1	0	2
Cpom-miR-3960	1	1	0	2
Cpom-miR-4608	1	1	0	2
Cpom-miR-7475	1	1	0	2

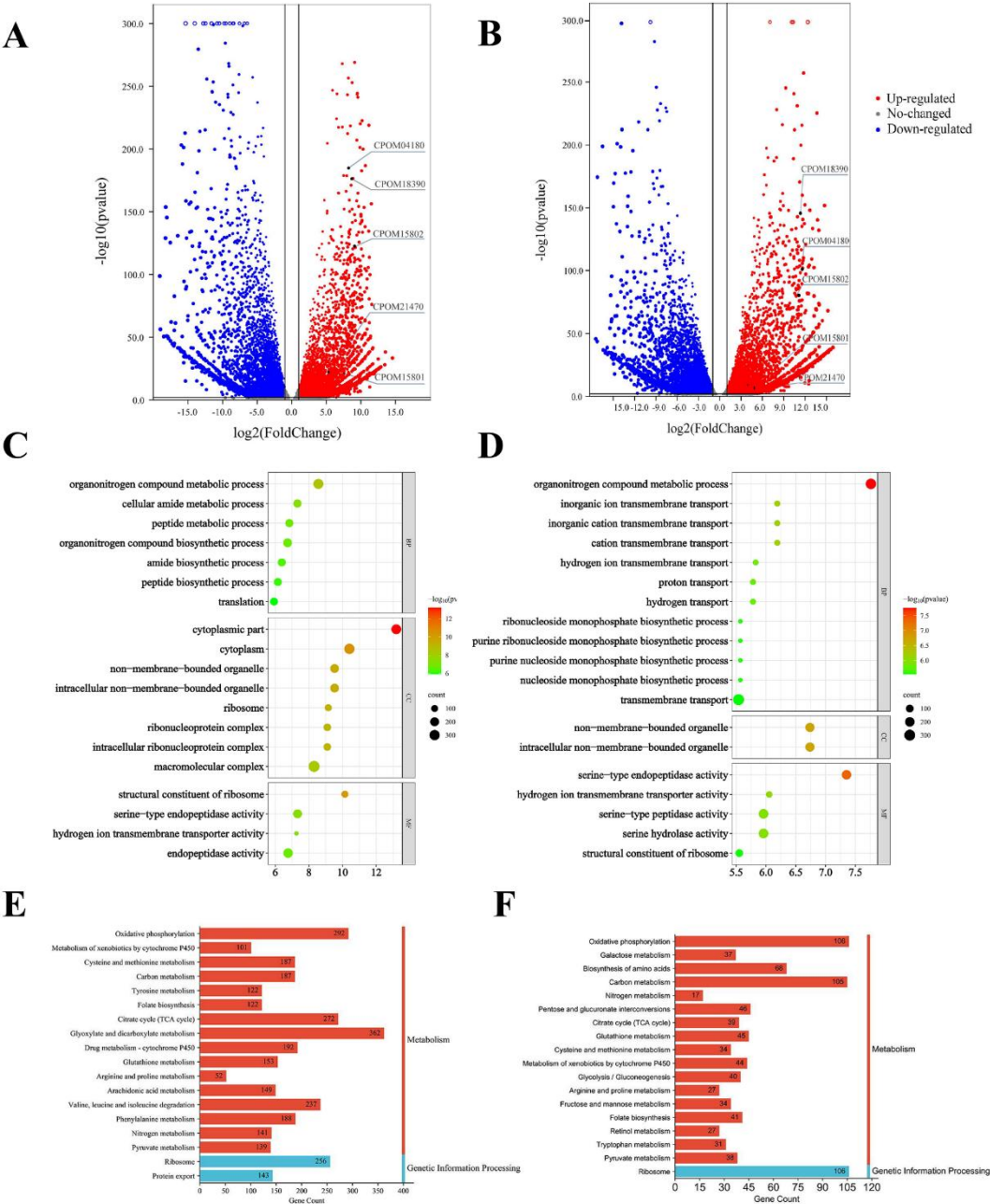
A value of 1 denotes the possibility of miRNA binding to lnc117962, whereas a value of 0 signifies the absence of binding.

Table S6. The prediction results of miR-3969-TSSKs information using different tools.

mRNA	RNAhybrid	PITA	miRanda	Total
TSSK1	0	1	0	1
TSSK1a	1	1	0	2
TSSK2	0	1	0	1
TSSK2a	1	1	1	3
TSSK4	0	1	0	1

miRNAs are capable of interacting with lnc117962 in instances where the interaction is signified by 1, and unable to form a binding when signified by 0.

Figure S1. Analysis of transcriptomic data from four tissues of codling moth. Volcano plots were utilized to illustrate the expression patterns of various genes in cpA2T versus cpA2U (A) and cpA5T versus cpA5U (B). The expression of different genes was further assessed through GO enrichment analysis in cpA2T versus cpA2U (C) and cpA5T versus cpA5U (D). Additionally, the KEGG enrichment analysis was employed to explore the expression of different genes in cpA2T versus cpA2U (E) and cpA5T versus cpA5U (F).



CpG Island ID	CpA2T	CpA2U	CpA5T	CpA5U
CPOM0103	16.00	0.00	16.00	0.00
CPOM15802	16.00	0.00	16.00	0.00
CPOM1690	16.00	0.00	16.00	0.00
CPOM15801	16.00	0.00	16.00	0.00
CPOM16327	0.00	0.00	0.00	0.00
CPOM1630	0.00	0.00	0.00	0.00
CPOM1673	0.00	0.00	0.00	0.00
CPOM16734	0.00	0.00	0.00	0.00
CPOM16713	0.00	0.00	0.00	0.00
CPOM1661	16.00	0.00	16.00	0.00
CPOM1616	0.00	0.00	0.00	0.00
CPOM16441	0.00	0.00	0.00	0.00
CPOM1726	0.00	0.00	0.00	0.00
CPOM1592	0.00	0.00	0.00	0.00
CPOM1422	0.00	0.00	0.00	0.00
CPOM16731	0.00	0.00	0.00	0.00
CPOM16025	0.00	0.00	0.00	0.00
CPOM11700	0.00	0.00	0.00	0.00
CPOM16089	0.00	0.00	0.00	0.00
CPOM1651	16.00	0.00	16.00	0.00
CPOM18513	0.00	0.00	0.00	0.00
CPOM1798	0.00	0.00	0.00	0.00
CPOM16084	0.00	0.00	0.00	0.00
CPOM1723	0.00	0.00	0.00	0.00
CPOM16063	0.00	0.00	0.00	0.00
CPOM169123	0.00	0.00	0.00	0.00
CPOM16292	0.00	0.00	0.00	0.00
CPOM12627	0.00	0.00	0.00	0.00
CPOM12865	0.00	0.00	0.00	0.00
CPOM13665	0.00	0.00	0.00	0.00
CPOM1639	0.00	0.00	0.00	0.00
CPOM1709	0.00	0.00	0.00	0.00
CPOM20073	0.00	0.00	0.00	0.00
CPOM1635	0.00	0.00	0.00	0.00
CPOM16934	0.00	0.00	0.00	0.00

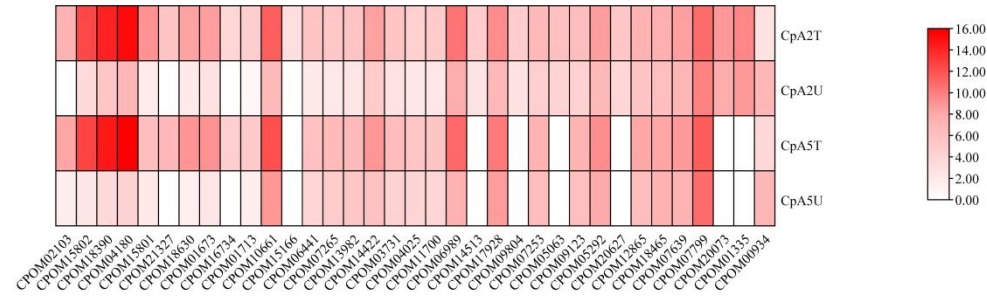


Figure S3. Transcription analysis of the expression patterns of tektin (A), cyclin (B), ubiquitin (C), tubulin (D) genes. The heat map illustrates normalized values of mRNA expression, varying from low (white) to high expression levels (red). All data underwent normalization using the log2 ratio.

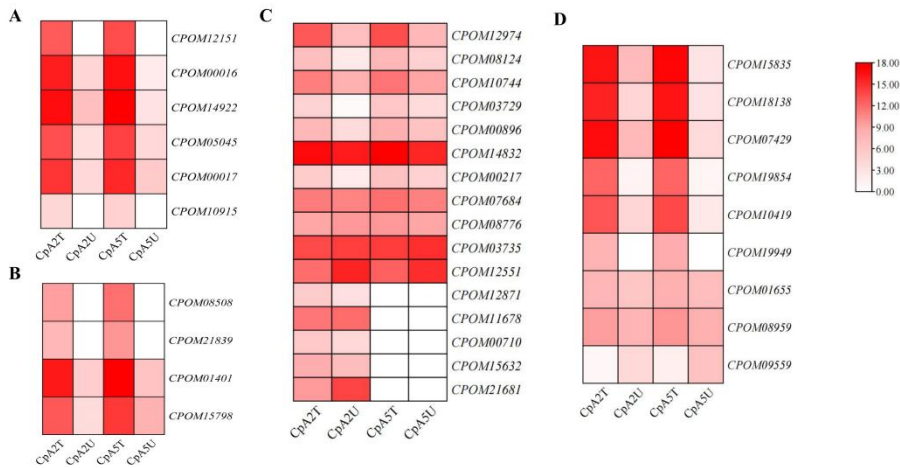


Figure S4. Relationship of TSSKs in Lepidoptera. Each representing a region where the homologous backbone sequence remains unchanged (collinear block).

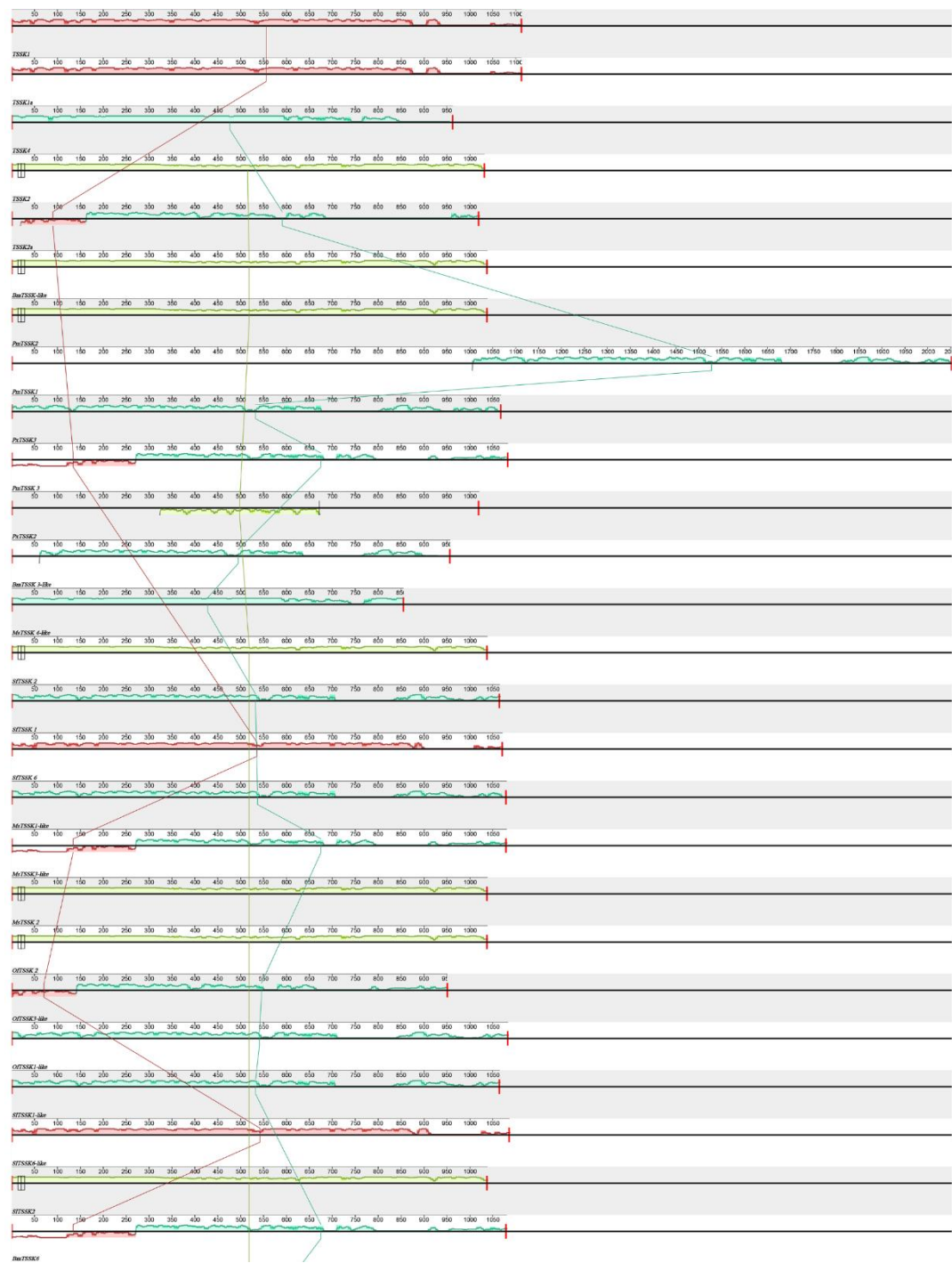


Figure S5. The relative expression levels of Testis-specific serine/threonine-protein kinase 2 (TSSK2) following RNA interference (RNAi) in pupae. The asterisks on the bars indicate the significant differences analyzed by the independent samples *t*-test. (* $P < 0.05$).

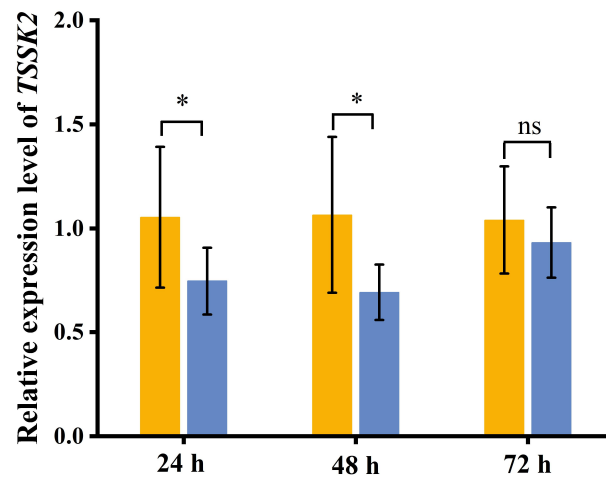


Figure S6. The morphology of the spermatophore in *C. pomonella* is indicative of mating. The presence of a spermatophore suggests mating has taken place, while its absence signifies that mating did not occur.

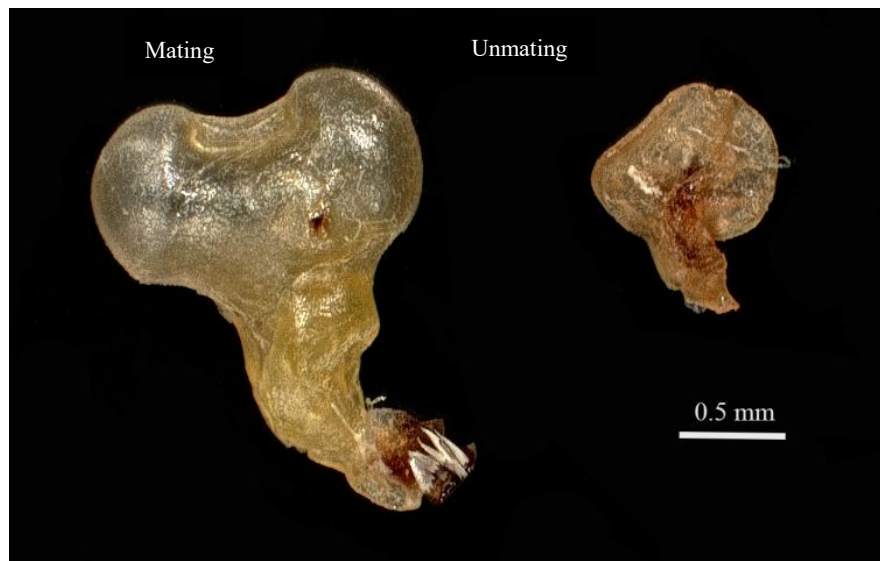


Figure S7. GO enrichment analysis of lnc117962 target genes.

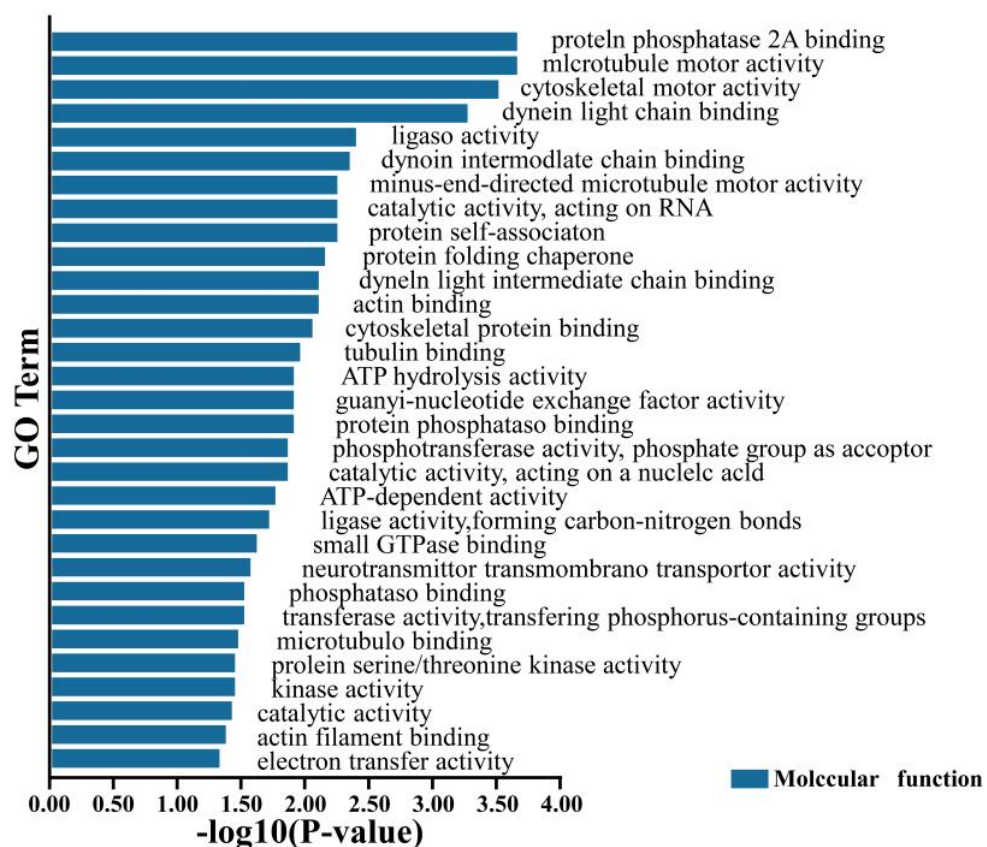


Figure S8. The impact of suppressing lnc117962 on the lifespan of male individuals. The t-test was employed to determine the significance of differences in dsGFP treatment between the dslnc117962 treatments.

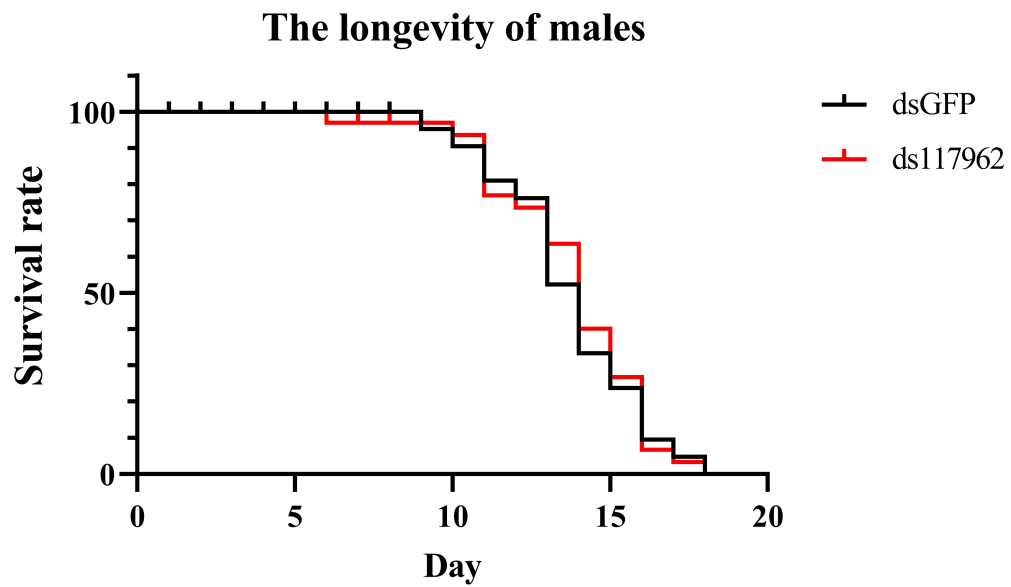


Figure S9. The weather conditions for June 2023 in Shenyang.

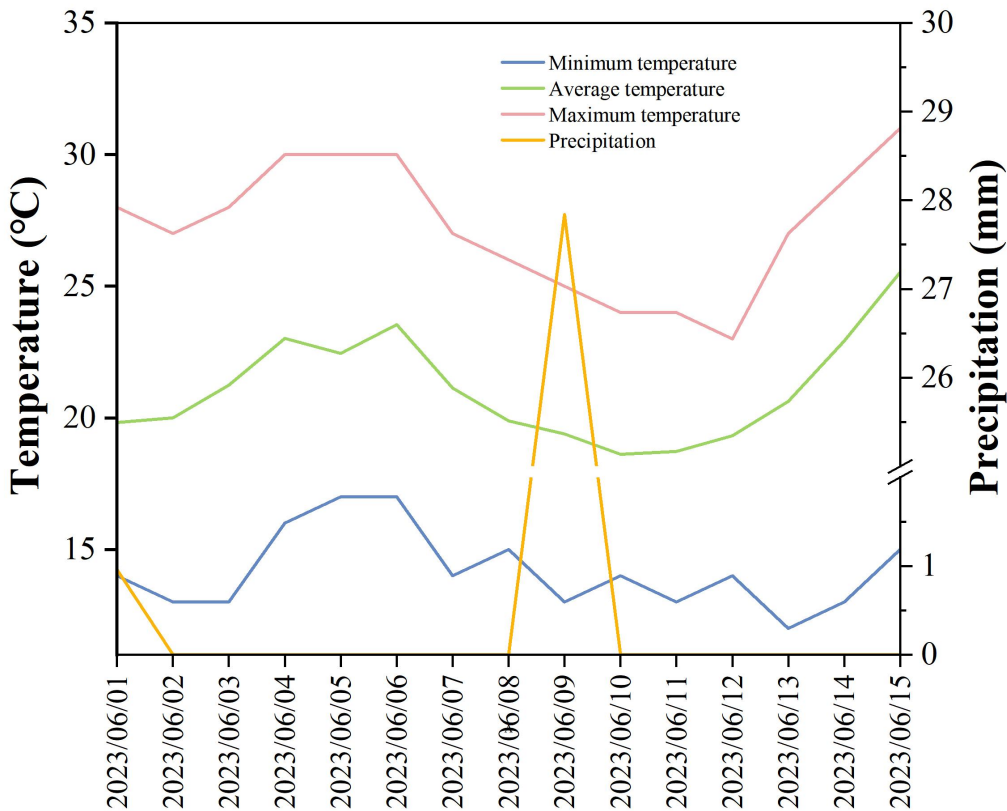


Figure S10. The impact of reducing lnc117962 expression on hatching rates in the first filial generation (F1). The asterisks on the bars indicate the significant differences analyzed by the independent samples t-test. (** $P < 0.01$).

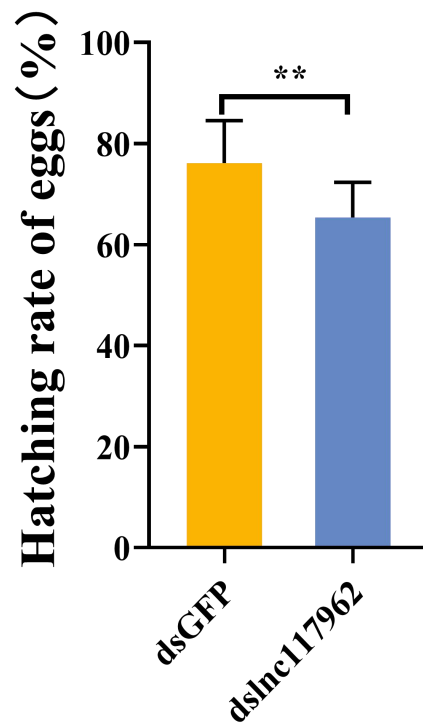


Figure S11. The relative expression levels of miRNAs in the knockdown of lnc117962 were analyzed. The asterisks on the bars indicate the significant differences analyzed by the independent samples t-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

