1 ***Nap1* is essential for eupyrene spermatogenesis and migration in *Plutella xylostella***

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27 **Abstract**

28 Spermatogenesis is a key process for the sexual reproduction species. In lepidopteran
29 insects, spermatogenesis produces two different types of sperms, in which eupyrene
30 sperm carry genomic DNA and fertilize eggs, whereas apyrene sperm are necessary for
31 eupyrene sperm to enter eggs. However, functional genetic studies of spermatogenesis
32 in *Plutella xylostella* remain a longstanding puzzle even though the phenomenon in
33 lepidoptera has been widely documented more than a century. In this study, we
34 particularly focus on the gene *Nap1* which belongs to the Nucleosome assembly protein
35 family. Our findings revealed that *Nap1* was highly expressed in the testes, and the
36 disruption of *PxNap1* induced male sterility in *P. xylostella*, while the fertility of mutant
37 females was comparable to wild-type females. Additionally, through
38 immunofluorescence staining analysis, we found that the eupyrene sperm bundles
39 presented diffusedly scattered nuclei in *PxNap1* mutant males, while the nuclei in the
40 wild-type were clustered together presented as needle shape. We also found that
41 *PxNap1* deficiency hinders the transfer of eupyrene sperm to the bursa copulatrix and
42 spermatheca of females. However, the apyrene spermatogenesis was not affected in the
43 *PxNap1* mutant. RNA-seq analyses indicated that the defects of eupyrene sperm in
44 *PxNap1* mutants were related to energy metabolic such as pentose and glucuronate
45 interconversions, biosynthesis of amino acids, and pentose phosphate pathway. Our
46 study demonstrates that *PxNap1* plays crucial function in eupyrene spermatogenesis
47 and eupyrene sperm migration. Our research provides valuable insights for the genetic
48 factors underlying reproductive processes in Lepidopteran insects.

49

50 **Keywords** *Plutella xylostella*; *Nap1*; spermatogenesis; male sterility; CRISPR/Cas9

51

52 # 1. Introduction

53 Sperms exhibit remarkable polymorphism, with a single male regularly producing
54 multiple distinct classes, which play a crucial role in sexual reproduction in the animal
55 kingdom (Hodgson, 1997). Dichotomous spermatogenesis is widespread from
56 invertebrates to vertebrates, including Arthropoda (Friedländer, 1997; Alberti, 2005),
57 Mollusca (Hodgson, 2000), Rotifera, and Chordata (Koehler et al., 1966; Hayakawa et
58 al., 2002). In insecta, several species of the genus *Drosophila* bear a form of sperm
59 dimorphism termed dimegaly, producing two size classes of nucleated sperm (Pasini et
60 al., 1996; Snook et al., 1998). In Lepidoptera, dichotomous sperm is a remarkable
61 feature in which two morphs of sperm exist in one male. Both sperm morphs are
62 essential for fertilization, eupyrene (nucleate) sperm fertilizes the egg, while apyrene
63 (anucleate) sperm is necessary for the migration of eupyrene sperm (Phillips, 1971).
64 However, little is known about the regulatory molecular mechanism underlying
65 dichotomous spermatogenesis.

66 Spermiogenesis is a highly dynamic process that involves the proliferation and
67 differentiation of spermatogonia, meiosis of spermatocytes, and spermiogenesis (Neto,
68 2016). Aberrant spermiogenesis negatively affects the quantity, motility, and
69 morphology of sperm, as well as the ability of sperm to fertilize the egg. Several factors
70 have been identified to regulate spermatogenesis and thus affect the reproduction of
71 male insects. For example, testis-specific serine/threonine kinases mediated
72 phosphorylation plays indispensable roles in *Drosophila* spermiogenesis (Zhang et al.,
73 2023). Protamine-like protein Mst77F plays a central role in spermatid nuclear shaping
74 during spermiogenesis (Doyen et al., 2015). *Sex-lethal* (*Sxl*) is essential for the
75 development of apyrene sperm bundles, and *Poly(A)-specific ribonuclease-like*
76 *domain-containing 1* (*Pnldc1*) regulates the development of eupyrene sperm in *Bombyx*
77 *mori* (Chen et al., 2020; Yang et al., 2022). We previously reported that *Serine Protease*
78 *1* (*SPSL1*), a key sperm activation factor in *Spodoptera frugiperda*, had affected the
79 structure of the spermatophore, sperm activation, and sperm migration in the female
80 reproductive tract (Qian et al., 2023). We also found *Protein arginine methyltransferase*

81 5 (*Prmt5*) and *Polyamine modulated factor 1 binding protein* (*PMFBP1*) are critical for
82 spermatogenesis in *B. mori* (Yang et al., 2022). Yet most studies on the mechanism of
83 dimorphic spermatogenesis have focused on model species *D. melanogaster* and *B.*
84 *mori*, the developmental genetics underlying the evolutionary diversity of sperm
85 remains largely unknown. Therefore, it's important to persist in investigating additional
86 genes in other insect species that may control dichotomous spermatogenesis, and thus
87 could provide a potential target gene for pest control.

88 Nucleosome assembly protein (Nap) is a kind of histone chaperon that plays a
89 critical role in histone trafficking, nucleosome assembly, and disassembly (De Koning
90 et al., 2007; Ransom et al., 2010). In *D. melanogaster*, *Nap1* and *Hanabi* mutants show
91 fully scattered nuclei and abnormalities in nuclear shaping during spermatid elongation
92 (Kimura, 2013). However, the physiological roles of *Nap* in other insect species are still
93 poorly understood. The diamondback moth (DBM), *Plutella xylostella* (Lepidoptera:
94 Plutellidae), is a worldwide pest of agricultural significance. It feeds mainly on
95 cruciferous plants, causing about $1 billion in losses annually in China (Li et al., 2016).
96 Its management is particularly challenging because of its high fecundity and
97 overlapping generations (Talekar et al., 1993; Furlong et al., 2013). However, the
98 molecular mechanisms involved in the reproduction of DBM are poorly understood. In
99 this study, we first identified the physiological function of the *Nap1* gene in DBM. We
100 observed that *PxNap1* is predominantly expressed in the testes of males, and loss-of-
101 function mutants of *PxNap1* exhibited significantly reduced fertility in males.
102 Immunofluorescent staining assays revealed that the *PxNap1* is indispensable for
103 eupyrene spermatogenesis and migration. Furthermore, RNA-sequence analysis
104 indicated a reduction in energy-related metabolites in the *PxNap1* mutant males.
105 Overall, our results shed light on the dichotomous spermatogenesis and reproductive
106 process of DBM, and provide a potential gene target for sterile insect technology.

107

108 ## 2. Materials and methods

109 ### *2.1 Insect strain and rearing*

110 The DBM was obtained from the Institute of Zoology, Chinese Academy of
111 Science (Beijing). The larvae were reared on an artificial diet of yeast powder,
112 raw wheat germ, and water at a temperature of 25 ± 1 °C, relative humidity of 65 ± 5%
113 and a photoperiod of 14 h: 10 h (L: D). The adults were fed with a 10% sucrose solution.

114 *2.2 Analysis and cloning of PxNap1 in DBM*

115 To identify the nucleosome assembly protein family genes homolog in DBM,
116 *Drosophila*'s Nap1, SET, CG3708 and Milkah protein sequences were used for
117 reciprocal best blast. To better illuminate the distribution and the evolutionary
118 relationship of the nucleosome assembly protein family in insect species, we
119 reconstructed a new phylogenetic tree using PxNap1 and 502 nucleosome assembly
120 protein family from insect species that have been reported at NCBI. All these protein
121 sequences were aligned using MUSCLE v3.8.31 (Edgar, 2004) and identified the
122 conserved blocks using Gblocks v0.91b (Talavera and Castresana, 2007). The
123 concatenated sequences of each species were used to determine the phylogeny using
124 RAxML (v. 8.0.19) (Stamatakis, 2014) under the PROTGAMMAAUTO model with
125 100 bootstrap replicates. All the protein sequences shown in Table S2.

126 Genomic DNA was extracted and purified from several pupae using a standard
127 extracted buffer and proteinase K (Sangon Biotech, China), and finally purified by
128 phenol: chloroform extraction and isopropanol precipitation, RNaseA. Polymerase
129 chain reaction (PCR) was conducted with 2×Hieff^{TM}PCR MASTER (YEASEN, China)
130 under the following conditions: 94 °C for 5 min, followed by 35 cycles at 94 °C for 30
131 s, 57 °C for 30 s and 72 °C for 2 min, and with an elongation step at 72 °C for 5 min.
132 Sequences of the RT-PCR primers are listed in Table S1. The amplified products were
133 confirmed by Sanger sequencing, and the results were compared with the *PxNap1*
134 genomic sequence to identify the exon-intron boundaries.

135 *2.3 Quantitative real-time PCR analysis*

136 Total RNA was extracted from the testes of mutant and wild-type (WT) moths that
137 had never mated to investigate the corresponding differential gene expression. Total
138 RNA was also extracted from legs, wings, head, fat body, midgut, ovaries and testes of
139 WT to investigate the expression patterns of *PxNap1*. Total RNA extracted from the

testes of the fourth instar larvae, prepupae, pupae, and adults was used to detect the expression quantity of *PxNap1*. RNA was extracted using the Trizol reagent (Invitrogen, USA) following the manufacturer's protocol. The RNA quality was checked with a spectrophotometer.

cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) from 1 μg total RNA. SYBR Green Real-time PCR Master Mix (Thermo Fisher Scientific, USA) was used for qRT-PCR performing Eppendorf Real-time PCR System Mastercycler RealPlex. To evaluate the quantitative variation, we used the $^{-\Delta\Delta}$Ct. All qRT-PCR experiments were performed in three independent biological replications. Ribosomal protein gene *S64* was used as the reference gene (Wang et al., 2021). Sequences of the qRT-PCR primers are listed in Table S1.

2.4 Mutant construction

A single guide RNA (5'- GGAGTTAGAGTCATCGGGCATGG-3') was designed at the third exon of the *Nap1* gene based on the $GGN_{19}GG$ rule. This single guide RNA was synthesized using the MEGAscript T7 kit (Ambion, USA) following the manufacturer's protocol in vitro. Cas9 mRNA was synthesized using the mMESSAGE mMACHINE Kit (Ambion, USA) in vitro according to the manufacturer's authentication protocol. The single guide RNA and the Cas9 mRNA were both purified with phenol: chloroform: isoamylol (25:24:1) and stored at -80 °C. The sgRNA and Cas9 mRNA were mixed at final concentrations of 300 ng/μL and 200 ng/μL, respectively. Fertilized eggs were collected and injected as described (Robinson et al., 2010). After injection, eggs were transferred into the incubator at 25 ± 1℃, 60 ± 5% relative humidity.

2.5 Mutation detection and phenotype observation

Randomly collecting the injected eggs, larvae or adults to extract the genomic DNA. Genomic regions around each of the sgRNA targets were amplified with specific primers (Table S1). Then the PCR products were recycled and used to ligate into the PMD18T vector and sequenced.

2.6 Fertility assay

170 To investigate whether *PxNap1* mutations resulted in male sterility, we performed 171 hatchability assays for mutant and WT moths. The injected eggs were reared to moths. 172 The WT male moths were respectively allowed to mate with WT and *PxNap1* mutant 173 females. Similarly, mutant male moths were respectively allowed to mate with WT and 174 *PxNap1* mutant females. All females were allowed to lay eggs for 24 h. The number of 175 eggs laid by different groups of females and the hatching rate were analyzed. Each test 176 was conducted with five pairs of moths and repeated 10 times

177 *2.7 Immunofluorescent staining*

178 Prior to insect dissection, ice was used to immobilize the insects. The testes were 179 dissected from the mutant and WT at different developmental stages in 1×PBS, then the 180 extra tissues were washed off by with 1×PBS. Testes were fixed using the Immunol 181 Staining Fix Solution (Beyotime, China), and a grinding rod was used to carefully 182 squish the testis, so that the sperm bundles could flow into the fix solution for 24h. 183 1×PBS was used to wash the sample thrice and each time for 5 min. Next, samples were 184 incubated with TRITC Phalloidin (YEASEN, China) for 1 h at room temperature. Then 185 samples were washed five times with 1×PBS. Hoechst33258 (Beyotime, China) was 186 used to stain the nucleus for 40 mins, then the samples were washed thrice in 1×PBS, 187 each time for 5 min. An OLYMPUS BX53 microscope (Japan) was used for fluorescent 188 imaging of the mutant and WT samples.

189 *2.8 Analysis sperm count*

190 Sperm bundles and sperms were collected from the testis, bursa copulatrix or 191 spermatheca of 4 h post copulation males and females. After fluorescent staining, the 192 samples were imaged using an OLYMPUS BX53 fluorescence microscope (Japan). 193 Eupyrene sperms were calculated by analyzing of 20× fields of view from 5 females 194 using the five-point sampling method.

195 *2.9 RNA-seq analysis*

196 Total RNA was extracted from the testes of *PxNap1* mutants and WT adults on the 197 first day after eclosion by using the Trizol reagent (Invitrogen, USA) following the 198 manufacturer's protocol. Twenty individuals were used for each sample, and three 199 independent replicates were performed. Then total RNA was first enriched and then

fragmented for cDNA synthesis and library construction. The Illumina 2000 platform was used to sequence the library, and FastQC was used to qualify the raw data, then the data were filtered by Trimmomatic. The filtered data were mapped and quantified to the reference *Plutella xylostella* database (http://iae.fafu.edu.cn/DBM/index.php). Differentially expressed genes (DEGs) between WT and *PxNap1* were normalized and analyzed by the DEGSeq R package (fold change > 1 and FDR < 0.05 were used as a cut-off) (Robinson et al., 2010). Enrichment analyses of DEGs were conducted using the gene ontology (GO) analysis with all the genes which were expressed in our study as a background set (Young et al., 2010). DEGs were functionally assorted and characterized using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8. All sequencing data have been deposited in GenBank under an accession code PRJNA1175736.

3.0 Data analysis

All the samples have at least three replications in this study. Statistical analysis was performed using SPSS 23.0 software with an independent Student's *t*-test. The data were presented as means ± SEM, and statistical significance was assumed at $P < 0.05$.

3. Results

3.1 Initial characterization of PxNap1

The *D. melanogaster Nap1*, *SET*, *CG3708* and *Milkah* ortholog were used as a query with blastn to identify the putative coding sequence of *Nap1* family genes from the DBM genome (You et al., 2013). In surprise, we identified a single ortholog gene in DBM, and no paralogs. The open reading frame from the putative *PxNap1* gene, consisting of 1215 bp, was amplified and cloned. By using the DBM genome and transcriptome database, we found the *PxNap1* transcript contains 8 exons and 7 introns, and encodes a putative protein with 405 amino acids (Fig. S1). Phylogenetic analysis of a total of 503 nucleosome assembly proteins from insect species resulted in four distinct clades and revealed that the Nap1 protein of DBM is clustered with the *D. melanogaster* Nap1 ortholog clade (Fig. 1). The amino acid sequences of Nap1 are

229 highly conserved among eight species of Lepidoptera including DBM, *B. mori*, *S.*
230 *frugiperda*, *S. exigua*, *S. litura*, *Chilo suppressalis*, *Helicoverpa armigera*, and *Pieris*
231 *rapae*. (Fig. S1).

232 To gain more insight into the function of *PxNap1*, its expression patterns were
233 investigated in moths at different tissues using qPCR. The results showed that *PxNap1*
234 is expressed at a higher level in the testis than in other tissues (Fig. 2A). The higher
235 expression of *PxNap1*, a kind of histone chaperone, in the testis may play an important
236 role in the development of testis tissue or sperm development. Meanwhile, to further
237 understand the temporal expression spectra, we quantified the relative expression levels
238 in the testes of the fourth instar larva, prepupae, pupae, and adults. The results showed
239 that *PxNap1* expression levels are higher in the testes of the prepupal and pupal stages
240 compared with other life stages (Fig. 2B). It may be because the sperm starts to develop
241 in these early stages, which proves that *PxNap1* plays a vital role in spermatogenesis.

242 *3.2 PxNap1 is essential for male fertility in DBM*

243 To explore the biological function of *PxNap1 in vivo*, we used the CRISPR/Cas9
244 system to obtain the loss-of-function mutant (Fig. 2C-F). To create a mutant, we
245 designed a targeted site in exon 3 of the *PxNap1* locus (Fig. 2C). Fresh eggs were
246 injected with sgRNA and Cas9 mRNA to obtain the mutant. Then, the *PxNap1* gene
247 was sequenced in 20 randomly injected eggs. These data revealed that mutations were
248 induced by Cas9/gRNA in targeted sites (Fig. 2D). TA cloning and sequencing showed
249 that diverse deletions or insertions occurred in *PxNap1* mutant individuals (Fig. 2F).
250 Analysis via qRT-PCR showed the expression of *PxNap1* in the mutants significantly
251 decreased compared with WT (Fig. 2E). These results demonstrated that we
252 successfully obtained the *PxNap1* mutant in DMB. The *PxNap1* mutants had viable and
253 normal surface phenotypes compared with WT DBM during all developmental stages.

254 To explore whether the disruption of *PxNap1* affected the fertility of DBM, we
255 examined the numbers and hatchability of eggs produced by WT and mutant moths. We
256 found that the fertility of the *PxNap1* mutant males was significantly reduced compared
257 to that of WT males (Fig. 3A-C). When mated with *PxNap1* mutant males, WT females
258 and *PxNap1* mutant females laid on average 97 and 87 eggs, respectively; when mated

259 with WT males, an average of 567 and 551 eggs were laid by WT females and by
260 *PxNap1* mutant females, respectively. In addition, mating with *PxNap1* mutant males
261 significantly reduced the hatching rate of laid eggs; the hatching rates of eggs laid by
262 WT females and *PxNap1* mutant females mated with *PxNap1* mutant males were only
263 36.36% and 37.75%, respectively. In contrast, when mated with WT males, an average
264 of 82.22% and 80.06% of eggs were hatched that were laid by WT females and by
265 *PxNap1* mutant females, respectively (Fig. 3A and C). To confirm the correlation
266 between phenotypic defects and mutations in *PxNap1*, we sequenced the target sites of
267 injected male adults, and detected several different kinds of mutagenesis events (Fig.
268 3F and G). Meanwhile, RT-PCR-based analysis revealed that *PxNap1* expression was
269 disrupted by using CRISPR/Cas9 (Fig. S2A). Taken together, these results
270 demonstrated that *PxNap1* is specifically essential for the fertility in males.

271 *3.3 PxNap1 is required for the euprene spermatogenesis in DBM*

272 The demonstration that *PxNap1* mutant males are sterile indicated possible defects
273 in the reproductive system. Therefore, we first investigated whether the *PxNap1* mutant
274 males exhibited any gross defects in external genitalia or internal genitalia system.
275 However, we confirmed that no significant defects were detected (Fig. 3D and E), so
276 we further searched for anomalies in spermatogenesis.

277 Sperm dimorphism has been reported in *B. mori* and *S. frugiperda* (Chen et al.,
278 2020; Yang et al., 2022; Qian et al., 2023). To date, there have been no reports
279 describing the morphology of sperm or the molecular mechanism of spermatogenesis
280 in DBM. Thus, to evaluate the morphology of sperm during spermatogenesis in WT
281 DBM, and to determine whether *PxNap1* is involved in spermatogenesis in DBM, we
282 performed fluorescence staining of sperm released from the testes of fourth instar larvae,
283 prepupae, pupae, and adult stages. We observed that the apyrene spermatogenesis starts
284 to show up in the late fourth larval stage while the eupyrene spermatogenesis mainly
285 occurs after the pupal stage in DBM (Fig. 4).

286 During spermatogenesis, the eupyrene sperm bundles are oval shaped and the
287 nuclei are like spots localized in the head in the early stage. With the development of
288 the cysts and the formation of the centriole as well as the mitochondrial derivative, the

289 sperm bundles grow longer with a wavy tail, and the nuclei become needle-like in shape
290 with a cyst-like elongation (Fig. 4B). The apyrene sperm bundles have scattered nuclei
291 in the spermatocyte, with the elongation of the apyrene sperm bundles, the nuclei in the
292 middle of the bundles were extruded out the sperm (Fig. 4C). In *PxNap1* mutants, the
293 apyrene sperm bundles appeared to present normal morphology in all life stages (Fig.
294 5B). Interestingly, we found that the nuclei were mingled and scattered in the head and
295 tail regions of eupyrene sperm bundles from *PxNap1* mutant testes (Fig. 5A and B),
296 while in the WT animals, the nuclei always gathered in the sperm's upper position in of
297 the sperm's head. In addition, we sequenced the target sites of male adults with
298 abnormal eupyrene sperm bundles, and detected several different kinds of mutagenesis
299 events (Fig. S2B). Thus, we deduced that a defect in *PxNap1* could influence the
300 eupyrene spermatogenesis of DBM.

301 *3.4 PxNap1 mutation affects the migration of eupyrene sperm*

302 After observing the morphological change of the two types of sperm, considering
303 the importance of sperm to animal reproduction, we next determined to explore whether
304 *PxNap1* mutation affects sperm transfer into the bursa copulatrix and spermatheca of
305 females. Hence, we then counted the sperm numbers in the testis of males, bursa
306 copulatrix and spermatheca of females at 4 h post copulation (hpc) (Fig. 6). We
307 observed that at 4 hpc the testis of *PxNap1* mutant males contained some abnormal
308 eupyrene sperm bundles, whereas the testis of WT males was almost empty (Fig. 6A
309 and A'). Remarkably, we found that the bursa copulatrix and spermatheca of females
310 mated with WT males contained abundant eupyrene sperm, whereas the number of
311 eupyrene sperm from the bursa copulatrix and spermatheca of females mated with
312 *PxNap1* mutant males was reduced drastically (Fig. 6B-C'). As expected, there were no
313 significant differences in the number of apyrene sperm in the bursa copulatrix and
314 spermatheca of females mated with WT or *PxNap1* mutant males (data not shown).
315 Meanwhile, we amplified and sequenced the *PxNap1* gene from male testis present
316 abnormal eupyrene bundles. We observed that a heterozygous chromatogram and
317 diverse deletions or insertions occurred in *PxNap1* mutant individuals (Fig. S2C). These
318 data implied that the developmentally arrested eupyrene sperm bundles failed to

transfer into the female reproductive organ from the *PxNap1* mutant males.

3.5 PxNap1 modulate the energy metabolism to influence the fertility of DBM

To further gain insights into the molecular mechanisms of the *PxNap1* mutant lead to defects in spermatogenesis and sperm migration, RNA-sequencing assay was carried out with the WT and the *PxNap1* mutant testes (eclosed moths at first day; mutation genotype results were shown in Fig. S2D). In *PxNap1* mutants, 354 differentially expressed genes were identified compared to WT. Among them, 61 were up-regulated and 293 were down-regulated (Fig. 7A). Among the genes significantly down-regulated by PxNap1, some are related to spermatogenesis, including those encoding Paired box protein 1 (Pax1), phospholipase A1 (PA1), Aminopeptidase N (APN), Vascular endothelial growth factor receptor 1 (VEGF), Adipokinetic hormone/corazonin-related peptide receptor (ACPR), Zonadhesin, AKR2E4 and Carboxypeptidase B (CPB) (Fig. 7B). Pax1, PA1 and VEGF are essential for sperm morphogenesis in mice (Oefelein et al., 1996; Nalbandian et al., 2003; Baba et al., 2014;). Zonadhesin, a sperm-specific membrane protein, functions in multiple cell adhesion processes in mice (Gao et al., 1998). APN modulates the motility trajectories of sperm in mice and humans (Khatun et al., 2017). PxACPR is crucial for development and reproduction in DBM (Pang et al., 2024). In *Bombyx mori*, the orthologs of the AKR2E4 and CPB play an important role in spermatogenesis (Yamamoto et al., 2017; Sakakura et al., 2022).

Next, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis and found that the pentose phosphate pathway, pentose and glucuronate interconversions, and biosynthesis of amino acids were dysregulated in *PxNap1* mutants (Fig. 7C). These three pathways are closely associated with energy metabolism (Britt et al., 2022; TeSlaa et al., 2023). Among them, the genes participating in energy metabolism are significantly down-regulated in *PxNap1* mutants, which include *ATPase subunit beta-1* (*ATPase*), *UDP-glucose 6-dehydrogenase* (*UDP6*), *mitochondrial uncoupling protein 4*, *mitochondrial dicarboxylate carrier*, *UDP-glycosyltransferase* (*UGT2* and *UGT5*), *phosphate dehydrogenase mitochondrial*, and *methylcrotonoyl CoA carboxylase β chain mitochondrial*, and it was verified that the expression of all these genes was dramatically decreased by 30%-60% in *PxNap1*

349 mutants compared to WT through qRT-PCR (Fig. 7D). Taken together, we concluded
350 that *PxNap1* takes part in the eupyrene spermatogenesis through the energy metabolism
351 to influence the fertility in the diamondback moth.

352

353 ## 4. Discussion

354 In *Drosophila*, *Nap1* mutation leads to abnormality of the nuclear shaping in the
355 later stage, while *Milkah*, another Nap family gene, mutant exhibits disruption of the
356 nuclear bundle at the early stage of spermatid elongation, and (Kimura, 2013; Xiao et
357 al., 2024). Interestingly, we demonstrated that early elongating eupyrene sperm bundles
358 appeared normal in the testes of *PxNap1* mutants; they exhibited disruption of the
359 nuclear bundle in the later stage. Notably, in our study, we found that the four
360 *Drosophila* paralogs (*Nap1*, *Milkah*, *SET* and *CG3708*), which belong to the Nap family
361 genes, were replaced by a single-copy ortholog *Nap1* in the genome of DBM (Fig. 1).
362 It's reported that the Nap1 protein was shuttled back and forth to neutralize the high
363 basic protein (Ito et al., 1996) and has functions in histone trafficking, nucleosome
364 assembly and disassembly (De Koning et al., 2007; Ransom et al., 2010). We therefore
365 speculate that other genes of DBM may compensate for the function of Nap family
366 genes in *Drosophila*, so further research is necessary to draw a firm conclusion.

367 In addition, we found that the *PxNap1* gene expression pattern at different stages
368 is consistent with the developmental stage of sperm bundles, that with the apyrene
369 spermatogenesis starting to show up in the late fourth larval stage and the eupyrene
370 spermatogenesis mainly occuring after the pupal stage in DBM. The expression level
371 of *PxNap1* was relatively low in the adult stage; we speculated that the one-day-old
372 adult moth is sexually mature, has completed most spermatogenesis and needs to mate
373 (Zheng et al., 2024).

374 During mating, males use spermatophores to transfer mating contents, such as
375 sperm, nutrients and seminal fluid, to the females (Gillott, 2003; Gwynne, 2008). The
376 contents of the spermatophore are transferred to the spermatheca of female moths,
377 where they mix with substances from the spermatheca, facilitating ovulation of the

378 female and completing the process of fertilization and production (Xu et al., 2020; Yang
379 et al., 2022). More importantly, in many insect species, females use nutrients from the
380 male's testis to support egg production (Friedländer et al., 1997; Yang et al., 2022, 2023).
381 Our research shows that the deletion of *PxNap1* not only caused a decrease in the
382 number of eupyrene sperm in the transfer from the male testis to the female moth's
383 bursa copulatrix during mating but also led to the down-regulated expression of energy
384 metabolism pathway genes, which has many effects on the subsequent reproductive
385 process (Fig. 8). Therefore, the drop in egg production is not only related to the lack of
386 eupyrene sperm, but may also be related to the disordered metabolism pathways
387 insufficiently providing enough nutrition for the females.

388 The Sterile Insect Technique (SIT) has been efficiently exploited for the
389 management of insect pests for more than 60 years (Knipling, 1955). The SIT
390 incorporates a radiation-based sterile technology (rSIT), microbe-mediated sterile
391 technology (mSIT), and/or genetic-based inheritable sterile technology (gSIT). gSIT as
392 a SIT strategy is becoming increasingly popular in recent years, with an improved
393 understanding of transgenic technology, TALENs and the CRISPR/Cas9 genome
394 editing system. Several cases of insect population control have been reported, such as
395 in *Drosophila melanogaster* (Thomas et al., 2000), *Ceratitis capitata* (Schetelig et al.,
396 2009; Enkerlin et al., 2015), *Aedes aegypti* (Midla, 2007), *Tribolium castaneum* (Xu et
397 al., 2012), and *Anopheles gambiae* (Kyrou et al., 2018). Genetic-based inheritable
398 sterile technology can compensate for the shortcomings in of high cost, repeat releasing,
399 mass rearing, and incompetent mating. Therefore, using genome editing techniques,
400 combined with the highly conserved *Nap1* gene is expected to develop a gene drive
401 system for controlling Lepidoptera pests. In conclusion, we identified *Nap1*, which is
402 highly conserved in the Lepidoptera, as being involved in regulating the eupyrene
403 spermatogenesis through the energy metabolism. Loss of function of *PxNap1* induced
404 male sterility due to the cytological and behavioral defects of eupyrene spermatozoa.
405 Thus, *Nap1* may be a potentially effective target to be explored in the gSIT. Our study
406 provides a novel target to be applied in the genetic regulation strategy to control
407 Lepidopteran pest populations.

CRediT authorship contribution statement

Chenxu Zhu: Validation, Software, Investigation, Data curation. **Lijun Zhou**: Validation, Software, Investigation, Data curation. **Dongbin Chen**: Methodology. **Xu Yang**: Investigation. **Lu Zhu**: Investigation. **Lansa Qian**: Data investigation. **Xiaomiao Xu**: Methodology, Investigation. **Gangqi Fang**: Methodology. **Awawing A. Andongma**: Writing – review & editing. **Yongcheng Dong**: Software. **Lin He**: Methodology. **Yongping Huang**: Writing – review & editing, Supervision, Funding acquisition. **Yaohui Wang**: Writing – review & editing, Writing – original draft, Validation, Supervision, Funding acquisition, Data curation.

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Data Availability

All relevant data are within the paper and its Supporting Information files.

Supplementary Information

Fig. S1 (A) Multiple alignment of Nap1 protein sequences from eight lepidopteran species.

Fig. S2 Targeted mutation of *PxNap1* induced by using the CRISPR/Cas9 system. **(A)** Related to Figures 3. **(B)** Related to Figures 5. **(C)** Related to Figures 6. **(D)** Related to Figures 7.

Table S1. Primers and sequenced in the study.

Table S2. Nucleosome assembly protein family sequences of all insects in Figure 1.

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588
589

590 # Figure legents

591 **Fig. 1. The maximum-likelihood phylogenetic tree of nucleosome assembly protein**
592 **families of insects.** Molecular phylogenetic analyses of Nap1, SET, CG3708 and
593 Milkah proteins were conducted from different insect species. Nap1, SET, CG3708, and
594 Milkah are shown by purple, pink, green, and blue frames, respectively. All the **protein**
595 sequences shown in Table S2.

596

597 **Fig. 2. Construction of *PxNap1* mutants using the CRISPR/Cas9 system.** (A)
598 Relative expression of *PxNap1* in seven tissues of 1-day-old DBM moth. Abbreviations:
599 WG (Wing), LG (Leg), HE (Head), MG (Midgut), FB (Fat body), OV (Ovaries), TE
600 (Testis). (B) Relative expression of *PxNap1* in four different stages. Abbreviations: L4
601 (Fourth instar larvae), PP (Prepupa), P (Pupa), A (Adult). mRNA expression was
602 normalized to *S64*. Three replicates were conducted. Bars with different letters above
603 them differ significantly at $P < 0.05$. The data shown are means ± S.E.M. (C) Mutant
604 target site designed in the sixth exon. Protospacer adjacent motif (PAM) sequences and
605 target sequences are highlighted in red and black, respectively. (D) Mutation sequence
606 in peak figure of the target. Red rectangle represents the mutation target site. (E) The
607 mRNA expression level of *PxNap1* in WT and mutant testes at adults. The three
608 asterisks indicate the significant differences ($P < 0.001$) relative to WT. (F) *PxNap1*
609 mutant sequences were confirmed by cloning and sequencing of the injected eggs.
610 Deleted bases are shown as dashes, insertions and PAM are highlighted in green and
611 red, respectively.

612

613 **Fig. 3. *PxNap1* mutations induce male sterility in DBM.** (A) The eggs laid by WT or
614 *PxNap1* mutant females mated with WT or *PxNap1* mutant males. Mut represents
615 *PxNap1* mutants. (B) The number of eggs laid for indicated crosses (Each test was
616 released with five pairs of moths and repeated 10 times. ***, $P < 0.001$. One-way
617 ANOVA test). (C) The hatching rate for indicated crosses (Each test was released with
618 five pairs of moths and repeated 10 times. ***, $P < 0.001$). (D) The morphology of

619 external genitalia of WT and *PxNap1* mutant moths. (E) The morphology of testis
620 dissected from WT and *PxNap1* mutant moths. (F) The representative sequencing
621 chromatogram run from injected males. Red rectangle represents the mutation target
622 site. (G) Various deletion mutations were detected in injected males. Dashed lines
623 represent the deleted bases, and PAM are highlighted in red.
624
625 **Fig. 4. Morphology of the dichotomic sperm bundles at different stages of**
626 **spermiogenesis in WT DBM.** (A) Representative immunofluorescence images of
627 spermatocysts of WT DBM. (B) Representative immunofluorescence images of the
628 eupyrene sperm bundles during the spermatogenesis of WT DBM. (C) Representative
629 immunofluorescence images of the apyrene sperm bundles during the spermatogenesis
630 of WT DBM. Red, TRITC Phalloidin; Blue, Hoechst33258. Scale bars, 50 μm.
631
632 **Fig. 5. Mutation of *PxNap1* influences the development of the eupyrene sperm**
633 **bundles.** (A) The mature eupyrene sperm bundles are abnormal in *PxNap1* mutant
634 moths. The nuclei are scattered around the head and tail region of each spermatozoon
635 in *PxNap1* mutants. The yellow arrows indicate the nuclei of eupyrene sperm bundles.
636 Red, TRITC Phalloidin; Blue, Hoechst33258. Scale bars, 50 μm. (B) The morphology
637 of apyrene sperm bundles of WT and *PxNap1* mutant moths. Red, TRITC Phalloidin;
638 Blue, Hoechst33258. Scale bars, 50 μm.
639
640 **Fig. 6. Mutation of *PxNap1* induces failure of sperm transfer into female**
641 **reproductive.** (A) Representative images of sperm bundles released from the testis at
642 4 hpc for indicated mating. The green arrows indicate eupyrene sperm bundles. Red,
643 TRITC Phalloidin. Blue, Hoechst33258. Scale bars, 50 μm. (A') The number of
644 eupyrene sperm bundles in the testis at 4 hpc for indicated mating. TE, testis. (B)
645 Representative images of eupyrene sperm released from the bursa copulatrix at 4 hpc
646 for indicated mating. The yellow arrows indicate eupyrene sperm. Blue, Hoechst33258.
647 Scale bars, 50 μm. (C) Representative images of eupyrene sperm released from the
648 spermatheca at 4 hpc indicated mating. The yellow arrows indicate eupyrene sperm.

649 Blue, Hoechst33258. Scale bars, 50 μm. (B'-C') The number of eupyrene sperm in the
650 bursa copulatrix (B') and spermatheca (C') after mating. BC, bursa copulatrix; SP,
651 spermatheca. 20× fields of view from 5 females for indicated mating were analyzed.
652 Data are means ± SEM (***, $P < 0.001$. two-tailed Student's *t*-test).
653
654 **Fig. 7. *PxNap1* modulate the eupyrene spermatogenesis through the energy**
655 **metabolism**. (A) Differentially expressed genes in the testes of WT and *PxNap1*
656 mutants. Red dots indicate the up-regulated genes, green dots denote the down
657 regulated genes, red and green number represents the number of regulated genes. (B)
658 Spermatogenesis-related genes with significant down-regulation in *PxNap1* mutant
659 testes. (FDR-adjusted $P < 0.05$). (C) KEGG enrichment analysis of the top 15 pathway
660 associated with the DEGs in *PxNap1* mutant and WT. (D) mRNA expression level of
661 *ATPase*, *UDP6*, *mitochondrial uncoupling protein 4*, *mitochondrial dicarboxylate*
662 *carrier*, *UGT2*, *UGT5*, *phosphate dehydrogenase mitochondrial*, and *methylcrotonoyl*
663 *CoA carboxylase β chain mitochondrial* dramatically decreased in *PxNap1* mutant
664 testes compared with WT. Data are mean ± SEM, determined by a two-tailed Student's
665 *t*-test (n = 3, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).
666
667 **Fig. 8. Model illustrating *PxNap1* mediated regulation of male fertility in DBM.**
668 Spermatogenesis and sperm migration are normal in the reproductive system of WT
669 (shown in orange boxes). In *PxNap1* mutant males, most of the eupyrene sperm bundles
670 had defects in sperm nucleus shape (blue box a). In the bursa copulatrix of females
671 mated with *PxNap1* mutant males, fewer eupyrene sperms are transferred and
672 dissociated during copulation (blue box b); thus, fewer eupyrene sperms are transferred
673 to the spermatheca for fertilization (blue box c). Abbreviations: TE, testis; BC, bursa
674 copulatrix; SP, spermatheca; Mut, *PxNap1* mutants.