Title: Testing for a role of *Dnmt2* in paternal trans-generational immune priming

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Abstract

Trans-generational effects from fathers to offspring are increasingly reported from diverse organisms, but the underlying mechanisms are often unknown. Paternal trans-generational immune priming (TGIP) was demonstrated in the red flour beetle *Tribolium castaneum*: non-infectious bacterial exposure (priming) of fathers protects their offspring against an infectious challenge. Here we studied a potential role of the *Dnmt2* (now also called *Trdnmt1*) gene, which encodes a highly conserved enzyme that provides CpG methylation to a set of tRNAs and has previously been reported to be involved in transgenerational epigenetic inheritance in mice. We first studied gene expression and found that *Dnmt2* was expressed throughout life, with high expression in testes. Knockdown of *Dnmt2* in fathers slowed down offspring larval development and increased mortality of the adult offspring upon bacterial infection. However, the observed effects were independent of the paternal priming treatment. In conclusion, our results point towards a role of *Dnmt2* for paternal effects, while elucidation of the mechanisms behind paternal TGIP needs further studies.

1 Introduction

Phenotypic plasticity is often enabled by epigenetic mechanisms [1,2]. An important epigenetic modification of nucleic acids is the covalent binding of a methyl group to a cytosine followed by a guanine, *i.e.* CpG methylation [3]. In insects, CpG methylation of DNA has been extensively studied [4,5] and is involved in many processes, such as caste determination and phase polyphenism [6–10]. However, this modification not only occurs on DNA, but also on a variety of RNAs[11]. CpG methylation is facilitated by a conserved family of enzymes called DNA methyltransferases, which are found in most but not all animals [3,12]. *Dnmt2* is the evolutionary most conserved member of this gene family. It can be found in many fungi, plant and animal species, sometimes occurring in the absence of any functional DNA methylation machinery [13]. While *Dnmt1* and *Dnmt3* are responsible for modifications of DNA, *Dnmt2* is involved in methylating three types of tRNA at the C38 position [14–16]. Because of this function, the human *Dnmt2* gene has been renamed to *TRDMT1* (tRNA aspartic acid methyltransferase 1). However, the gene is phylogenetically related to *Dnmt* genes, and it was thus suggested to stick to its original name[3]The methylation mark provided by *Dnmt2* protects the tRNA molecule against cleavage, which can be induced by different stressors [15]. It has been shown that tRNA-derived small RNAs (tsRNAs) regulate mRNAs and therefore differences in tRNA cleavage could lead to altered phenotypes. In mice dietary stress can cause increased fragmentation of tRNAs and the resulting metabolic phenotype is paternally transmitted to the offspring through the altered levels of tsRNAs [17,18].This paternal transmission is dependent on *Dnmt2*, which demonstrates the importance of this gene in non-genetic inheritance [19,20].

The function of *Dnmt2* has also been studied in *Drosophila melanogaster*. Mutants lacking *Dnmt2* were less protected against a variety of stressors, as increased rearing temperatures led to a reduced lifespan and herbicides caused higher mortality compared to wildtype and control flies [15]. Furthermore, heat shock treatment of flies lacking *Dnmt2* led to the accumulation of transposable elements and changed gene expression [21]. Also, other studies have demonstrated that *Dnmt2* plays a crucial role in managing endogenous and exogenous RNA stress, by silencing retrotransposons and inhibiting RNA

virus replication [22,23]. It has thus been proposed that the enzyme is involved in adaptive immunity and aids in defending against or adapting to pathogens [13].

Phenotypic plasticity, both within and across generations, is widespread in immune defence. A wealth of studies in invertebrates now provide evidence for immune priming, i.e. enhanced survival upon a secondary encounter with a pathogen [24–26]. In some species it has been shown that the immune priming can also be transferred to the offspring [25,27,28]. While maternal transfer appears to be a relatively common phenomenon, reports about paternal transgenerational immune priming (TGIP) are scarce [25,29]. The red flour beetle, *Tribolium castaneum* is the first example where paternal TGIP against a variety of bacterial pathogens has been demonstrated [29–31]. However, the mechanisms underlying TGIP remain elusive. The paternal route of priming narrows down the possibilities by which effectors or information could be transferred from father to offspring and thus makes the involvement of epigenetic modifications, such as methylation of sperm RNA, especially likely [26]. Finally, in another beetle, *Tenebrio molitor* priming of adults and larvae decreased overall RNA methylation within the generation, hinting at a possible involvement of *Dnmt2* [32].

T. castaneum possesses two Dnmt genes: one *Dnmt1* and one *Dnmt2* homolog [33]. The beetle seems to lack any functional levels of CpG DNA methylation [4,34,35], but *Dnmt1* is nevertheless expressed across all life stages and is needed for proper embryonic development [35]. To our knowledge no research has been dedicated yet to study the role and function of *Dnmt2* in *T. castaneum*. We therefore used gene expression analysis and RNAi to investigate the role of this gene for paternal trans-generational effects. We combined a paternal knockdown with immune priming, to investigate whether *Dnmt2* is involved in and possibly provides the epigenetic mechanism behind paternal TGIP.

2 Materials and methods

2.1 Model organism

T. castaneum has become a well-established model organism in many fields of biology including evolutionary ecology. The availability of a fully sequenced genome [33] and molecular tools, *e.g.* easily established systemic RNAi and genome editing [36–39] allow for genetic analyses of ecologically relevant phenomena. For this study, the *T. castaneum* line Cro 1 was used, which was established from 165

84 pairs of wild caught beetles collected in Croatia in June 2010 [40]. Beetles were maintained in plastic
85 breeding boxes with foam stoppers to ensure air circulation. Standard breeding conditions were 30°C
86 and 70% humidity with a 12-hour light/dark cycle. As food source 250g of heat sterilized (75°C)
87 organic wheat flour (type550) containing 5% brewer's yeast were given.

88 2.2 Gene expression of *Dnmt2*

89 To assess the expression of *Dnmt2* throughout the life cycle of the beetle, the four distinct life stages
90 were sampled: eggs (n=4 pools of 500-1000 eggs, 24h-48h post oviposition), larvae (n=7 pools of 10
91 larvae, 14-19 days post oviposition (dpo)) pupae (n=8 pools of 6 individuals), virgin adults (n=8 pools
92 of 6 individuals, one week after eclosion). For pupae and adults, half of the pooled samples contained
93 females and the other half males in order to test also for differential expression between the sexes.
94 Furthermore, gonads were dissected from unmated adult males. All samples were shock frozen in
95 liquid nitrogen. Total RNA was extracted, and genomic DNA digested by combining Trizol (Ambion
96 RNA by Life Technologies GmbH, Darmstadt, Germany) and chloroform treatment with the use of
97 the Total RNA extraction kit (Promega GmbH, Mannheim, Germany) as described in Eggert *et al.* [30].

98 Extracted RNA was reverse transcribed to cDNA with the RevertAid First Strand cDNA kit (Thermo
99 Fisher Scientific, Waltham, MA USA) using provided oligo-dTs. In the following RT qPCR with a
100 Light-Cycler480 (Roche) and Kapa SYBR Fast (Kapa Biosystems, Sigma-Aldrich), each sample was
101 used in two technical replicates. Further analysis was conducted as described in Eggert *et al.* [30] and
102 replicates were used in further analysis if the standard deviation between their crossing point values
103 was below 0.5, otherwise the reaction was repeated. Previously, high primer efficiency had been
104 confirmed and where possible it was made sure that primers crossed exon-intron boundaries (Table
105 S1). The housekeeping genes ribosomal proteins rp49 and rpl13a were used for normalization of the
106 expression of the target genes.

107 2.3 Paternal *Dnmt2* knockdown and TGIP

108 We aimed to downregulate *Dnmt2* through paternal RNAi and to investigate whether this knockdown
109 would affect paternal TGIP (Figure 1). For this, around 2000 adult beetles (one week old) were allowed

to lay eggs for 24h. Two weeks later, larvae were collected and put into individual wells of a 96 well plate, which contained flour and yeast. The oviposition was repeated with two further, independent cohorts on the two following days, producing three experimental replicates.

2.3.1 Paternal RNAi

The sex of the beetles was determined in the pupal stage, and male pupae were prepared for RNAi treatment, while females were individualized and kept for mating. For injections of dsRNA, male pupae (22 dpo) were glued with the hindmost segment of the abdomen to a glass slide to immobilize them. One glass slide held between 16 and 20 pupae. Pupae were either injected with dsRNA of the target gene *Dnmt2* or for the control of the treatment procedure with dsRNA transcribed from the *asparagine synthetase A* (asnA) gene found in *Escherichia coli* (RNAi control), which bears no sequence similarity to any known *T. castaneum* gene (34, Table S1). The dsRNA construct for the RNAi control was produced in our lab via cloning followed by PCR and *in vitro* transcription using the T7 MEGAscript Kit (Ambion by Life TechnologiesTM GmbH, Darmstadt, Gemany) [38]. The *Dnmt2* dsRNA construct has been previously used in the ibeetle RNAi scan (33; http://ibeetle-base.uni-goettingen.de/details/TC005511) and was obtained from EupheriaBiotech (Dresden, Germany). Injections were carried out with a microliter injector (FemtoJet, Eppendorf AG, Hamburg, Germany) and borosilicate glass capillaries (100 mm length, 1.0 mm outside diameter, 0.021 mm wall thickness; Hilgenberg GmbH, Malsfeld, Deutschland) using dsRNA at a concentration of 1000 ng/µl dissolved in ultrapure water. We injected pupae between the second and third lowest segment of their abdomen.

Over the three experimental blocks a total of 583 pupae were injected with *Dnmt2* dsRNA and 585 pupae served as RNAi control and were therefore injected with *asnA* dsRNA. Eclosion and survival of the procedure were recorded daily from three to six days post injection.

2.3.2 TGIP

When all surviving males from the RNAi treatment had reached sexual maturity seven days after eclosion, they were injected for priming with around 37,000 cells of heat-killed *B. thuringiensis* (DSM no. 2046, obtained from the German Collection of Microorganisms and Cell Cultures, DSMZ)

suspended in phosphate buffered saline (PBS). This treatment has successfully been used in prior TGIP experiments [29,30]. Bacterial cultures were grown overnight as previously described [41]. They were washed with PBS and heat-killed by exposure to 95°C for 30 minutes. Control groups were either injected with PBS (injection control) containing no bacterial cells or were left naïve. Injections were performed using the nanolitre injector Nanoject II (Drummond Scientific Company, Broomall, PA, USA) and individuals were injected between head and thorax. Beetles were kept individually before and after the injections. Survival of the priming procedure was recorded 24h later.

2.3.3 Gene expression after RNAi and priming treatment

Twenty-four hours post priming, a subgroup of males was used for gene expression analysis to confirm the knockdown of *Dnmt2*. In addition to the expression of *Dnmt2*, the expression of three immunity and stress-related genes (*hsp83*, *nimB* and *PGRP*; Table S1) was analyzed, based on a previous study showing effects of paternal priming on gene expression [30]. For each RNAi*priming treatment combination and block five samples were taken consisting of a pool of two to five individuals. RNA extraction, cDNA reverse transcription and RT qPCR were performed as described above (2). Finally, we also analyzed the expression of seven transposable elements (TEs) (Table S1), because the absence of *Dnmt2* can cause the activation of TEs [21]. Because of the lack of polyadenylation of some TE transcripts, we used random hexamer primers for cDNA reverse transcription in this case (Thermo Fisher Scientific, Waltham, MA USA).

2.3.4 Production and development of offspring generation

One day after the priming procedure, single pair matings were carried out for 24h with virgin females from the same population (n=12-50 mating pairs per treatment combination and experimental replicate). Twelve days after the oviposition to produce the F1 offspring generation, larvae from each pair were counted and up to six individuals were individualized and kept for further analyses. Additionally, one larva from each mating pair that produced offspring was used for developmental checks until it died or eclosed as an adult. The development was monitored daily from 21 to 23 dpo to check for pupation and at 26 dpo we recorded the proportion of eclosed adults.

2.3.5 Gene expression in the offspring generation

One week after the majority of the offspring generation had eclosed, five pools per RNAi and priming treatment combination and experimental replicate were sampled for gene expression analysis. Each sample consisted of five adult beetles of unknown sex. To avoid pseudo-replication only one beetle per family was used. Again, the expression of *Dnmt2* and three potential TGIP marker genes (*hsp83*, *nimB*, *PGRP*; 26) was analyzed as described above (3).

2.3.6 Bacterial challenge of adult offspring

One week after their eclosion, adults of the F1 offspring generation were submitted to a potentially lethal bacterial injection (challenge). For this, bacteria from the same *B. thuringiensis* stock as for the priming were used. Again, an overnight culture from a glycerol stock was grown in liquid medium and washed in PBS. The injection procedure was similar to priming and again included an injection control and a naïve group. The dose was adjusted to around 370 live bacterial cells per animal. From every family one sibling each was used for the treatment and the controls. Again, beetles were kept individually before and after injection to avoid any cross contaminations. Survival of the challenge was recorded one day and four days post injection.

2.4 Statistics

All gene expression data were analyzed with the REST2009 software [42] as described in Eggert *et al.* [30]. All other analyses were performed in RStudio version 0.99.467 [43] under R version 3.3.3 [44] using additional packages lme4 [45] and MASS [46].

Survival of injections for RNAi and priming in the parental generation, the fertility of the treated males as well as the development of the offspring (proportion of pupae 21-23 dpo and proportion of adults 26 dpo) and their survival after bacterial challenge were analyzed in generalized linear mixed effect models (GLMMs) with the according error distributions and experimental replicate as a random factor.

3 Results

3.1 Expression of *Dnmt2*

Before investigating a possible function of *Dnmt2* in *T. castaneum*, we monitored expression of this gene throughout the life cycle of the beetle (i.e. in eggs, larvae, pupae and adults). The levels of *Dnmt2* transcripts (relative to two housekeeping genes) in eggs and pupae closely resembled those in adults (eggs: relative expression=0.932, n=4, p=0.76; pupae: relative expression=0.989, n=8, p=0.94; Figure 2a). Larvae expressed detectable amounts of *Dnmt2*, but significantly less than adults (relative expression=0.352, n=7, p<0.001; Figure 2a). Additionally, *Dnmt2* appears to serve functions in both males and females as its expression did not differ significantly between the sexes for pupae (female: relative expression=0.784, n=4, p=0.23) or adults (female: relative expression=0.709, n=4, p=0.14).

Furthermore, we analyzed the expression of *Dnmt2* in the reproductive tissue of the male beetles and compared it to whole body samples of the same sex, because expression in the testes could hint to a possible relevance of the protein in male reproduction or even an involvement in the transfer of information from father to offspring as possibly needed for TGIP. *Dnmt2* mRNA levels in the testes were significantly higher than in whole-body samples (relative expression=2.497, n=6, p=0.001; Figure 2b).

3.2 Paternal *Dnmt2* knockdown and TGIP

To determine whether *Dnmt2* is involved in the paternal transfer of immunity, we combined a knockdown with paternal TGIP treatment and exposed the offspring to a bacterial challenge (Figure 1).

3.2.1 Survival of RNAi and priming injections

The RNAi treatment with *Dnmt2* dsRNA did not increase mortality of the treated pupae (Figure S1). Injections of dsRNA in male pupae did not significantly alter survival rates neither directly following the RNAi treatment (GLMM, df=1, X^2=0.04, p=0.84, Figure S1a) nor after the priming treatment in the mature adults ten days later (GLMM, df=1, X^2=0.16, p=0.69; Figure S1b). The priming procedure itself led to a significantly increased mortality. However, the effect size was small and independent of whether the beetles were injected with heat killed bacteria or the PBS treatment control, which can be

attributed to the wounding during these injections as none of the naïve individuals died (GLMM, df=2, X^2=15.89, p<0.001; Figure S1b).

3.2.2 Knockdown of *Dnmt2*

One day after the priming procedure, we confirmed the knockdown of *Dnmt2* after pupal RNAi in a subgroup of the adults. *Dnmt2* was significantly downregulated compared to RNAi control regardless of the received priming treatment (Table 1). As expected, *Dnmt2* mRNA levels had returned to normal in the adult offspring and there were no significant differences between the RNAi treatments detectable (Table 1). Additionally, the paternal priming procedure did not affect *Dnmt2* expression in the adult offspring (Table 1).

3.2.3 Knockdown of *Dnmt2* and adult priming did not affect male fertility

Neither the knockdown of *Dnmt2* nor the bacterial priming appear to affect the fitness of the treated individuals, as neither treatment significantly altered male fertility. The number of live offspring obtained from a 24 h single pair mating period did not differ significantly for either of the treatments (GLMM: RNAi, df=1, X^2=2.11, p=0.15; priming, df=2, X^2=0.44, p=0.80; Figure S2).

3.2.4 Paternal knockdown of *Dnmt2* but not priming affected offspring development

We monitored offspring development by measuring the proportion of pupae over three consecutive days and the proportion of eclosed adults 26 days post oviposition (dpo). Animals from all six treatment combinations of RNAi and priming showed similar pupation rates 21 and 22 dpo (Figure 3a, Figure S3a). However, at 23 dpo significantly less larvae had reached pupation in the *Dnmt2* paternal knockdown group than in the RNAi control, independent of paternal priming treatment (GLMM: RNAi, df=1, X^2=3.9, p<0.05; priming, df=2, X^2=0.19, p=0.91; Figure 3a). The proportion of eclosed adults 26 dpo was not significantly affected by any paternal treatment (GLMM: RNAi, df=1, X^2=0.04, p=0.84; priming, df=2, X^2=0.48, p=0.79; Figure S3b).

3.2.5 Expression of TGIP marker genes and TEs is not affected by *Dnmt2* knockdown or priming

In fathers and offspring alike, we measured the expression of three genes, which are related to stress or immune responses and were previously shown to be upregulated in the adult offspring of primed fathers (26). By measuring the expression in the fathers, we intended to see whether these genes would already be affected within the treated generation. None of the three candidate genes (*hsp83*, *nimB* and *PGRP*) showed any significant differential expression neither in the paternal nor in the adult offspring generation (Table S2).

For the same animals from the paternal generation we also measured the expression of seven TEs. Genenncher *et al.* [21] observed that the absence of *Dnmt2* and the exposure to heat stress led to the activation and accumulation of certain TEs in *D. melanogaster*. Here, we could not observe any significant upregulation in the expression of TEs after wounding or priming in the knockdown or control treatment (Table S3).

3.2.6 Paternal *Dnmt2* knockdown reduces offspring survival after bacterial challenge

Finally, we injected adult beetles from the offspring generation with a potentially lethal dose of *B. thuringiensis* to see whether the immune priming was transmitted to the offspring and if this was affected by the downregulation of *Dnmt2* in the fathers. Paternal priming treatment did not affect offspring survival after bacterial challenge (GLMM, df=2, X^2= 0.17, p=0.92; Figure S4), which can possibly be explained by the unavoidable additional wounding that all fathers were subjected to because of the RNAi injection treatment. However, offspring of individuals that had received a knockdown were significantly less likely to survive the bacterial challenge (GLMM, df=2, X^2=7.78, p<0.01; Figure 3), demonstrating that *Dnmt2* is involved in paternal effects and that its reduction can increase the offspring's susceptibility to pathogens in the beetle.

4 Discussion

Dnmt2 can be found in almost every eukaryote species and is the most conserved member of the Dnmt family [47]. It also has a function in some organisms lacking one or both other Dnmts and which are often devoid of any functional DNA methylation system [13]. This also appears to be the case in *T. castaneum*, which lacks *Dnmt3* and does not have any functional CpG DNA methylation [4,33–35], but

still expresses *Dnmt2*. Although, we know today that *Dnmt2* methylates tRNA and not DNA, it remains unclear what the role of this epigenetic mechanism is. We observed that *Dnmt2* mRNA transcripts are present at relatively low but consistent levels in all life stages and in both sexes of the beetle, therefore the enzyme likely functions in both males and females and throughout the entire life cycle. In fruit flies, mice and humans, *Dnmt2* canonically methylates a small set of tRNAs [14,15,48], which are highly abundant in sperm [49] and have been shown to be involved in paternal transmission of metabolic phenotypes in mice [17,18]. The significantly higher expression of *Dnmt2* that we observed in testes of *T. castaneum* could hint at the involvement of *Dnmt2* in paternal epigenetic inheritance also in this beetle.

We used systemic paternal RNAi, to investigate how a *Dnmt2* knockdown would affect offspring phenotypes and to discover a potential role of this gene in intergenerational epigenetic inheritance, specifically from father to offspring. The offspring of *Dnmt2* RNAi-treated fathers needed longer to reach pupation and were less well dealing with a *B. thuringiensis* infection, which may point towards a generally higher stress sensitivity. In recent years, it has become clear that biological functions of *Dnmt2* are more easily detected under stress conditions [13]. Increased sensitivity to thermal and oxidative stress has been observed in *D. melanogaster Dnmt2* mutants [15], while overexpression of the same gene led to increased stress tolerance [50]. During the stress response, *Dnmt2* appears to control the fragmentation of tRNA and can be located at cellular stress compartments [15,51]. More recently, it has also been demonstrated that the knockout of *Dnmt2* leads to a decline of immune function with ageing in adult flies [52]. Finally, its absence disrupts the small interfering RNA pathway by inhibiting dsRNA degradation by *Dicer* [51]. However, all these effects occur in the treated generation and have not been tested for an offspring generation. In our case the developmental and immunological effects were observed in the offspring of treated fathers, even though offspring themselves exhibited normal *Dnmt2* expression, which suggests transgenerational effects. A role in transgenerational paternal effects for *Dnmt2* has so far only been established in mice, where the gene is essential for the transmission of an acquired metabolic disorder [19]. Therefore, further studies are needed to clarify the role that *Dnmt2* plays in enabling appropriate stress responses within and across generations.

289 Finally, we combined the knockdown of *Dnmt2* with a TGIP treatment, to determine whether this
290 enzyme is involved in the transfer of priming information from father to offspring. We did not observe
291 any TGIP in the present study, as offspring survival was independent of the paternal priming treatment.
292 Furthermore, we did not observe any upregulation in the previously described marker genes for
293 immune and stress responses [30] or any changes in the expression of transposable elements. This
294 surprising lack of any observable difference between offspring of primed and control males might have
295 been caused by the injection of all males for the pupal RNAi treatment that took place in the pupal
296 stage, before the priming treatment in the adult stage (note that we included an RNAi control, but did
297 not have a fully naïve control for the RNAi treatment, Fig. 1). The wounding associated with the
298 dsRNA injection might have activated immune responses, such that all animals could already have
299 entered a 'primed' state. To our knowledge there are no studies that directly address the question how
300 injuries during the pupal phase influence later immune responses. However, some experiments show
301 that wounding in control treatments also to a certain extent may increase survival of a subsequent
302 bacterial challenge [29,53]. Alternatively, the pupal RNAi injections might have inhibited priming. Lastly,
303 although TGIP in *T. castaneum* is robust and repeatable [29,30,53], it also has become apparent that this
304 phenomenon cannot be observed in every experiment [53] and beetle population [54].

305 We did not observe any effects of the Dnmt2 knockdown on expression of the studied TEs, in contrast
306 to *D. melanogaster Dnmt2* mutants that showed increased TE expression [21]. However, further studies
307 would be needed to make any firm conclusions, because the lack of expression differences for a limited
308 set of TEs does not exclude the possibility that *Dnmt2* plays a role in the regulation of TEs in *T.*
309 *castaneum*.

310 In plants, flies and mice the absence of *Dnmt2* is not lethal under standard conditions and mutants
311 remain fertile [14]. The same appears to be true in the case of *T. castaneum*, where we did not observe
312 any mortality nor apparent phenotypic changes after a significant downregulation of *Dnmt2*.
313 Additionally, male fertility was not affected by the knockdown under *ad libitum* conditions. Therefore,
314 the maintenance of knockout lines appears feasible, which makes this gene a suitable target for
315 CRISPR/Cas knockout to further study its function without the necessity of repeated RNAi injections

for each experiment. Moreover, methylation-sensitive sequencing of sperm RNA could further elucidate the underlying molecular processes.

In conclusion, our study for the first time describes paternal effects of *Dnmt2* in an invertebrate. The here observed prolonged development and increased susceptibility to infection in the offspring occurred in the presence of normal *Dnmt2* expression in the offspring themselves. Therefore, tRNA methylation in sperm provides a fascinating possibility of transmitting information from fathers to offspring through paternal epigenetic inheritance.

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Contributions

All authors conceived and designed the experiments. NS conducted the experiments, analyzed the data, and wrote the manuscripts with comments from all authors.

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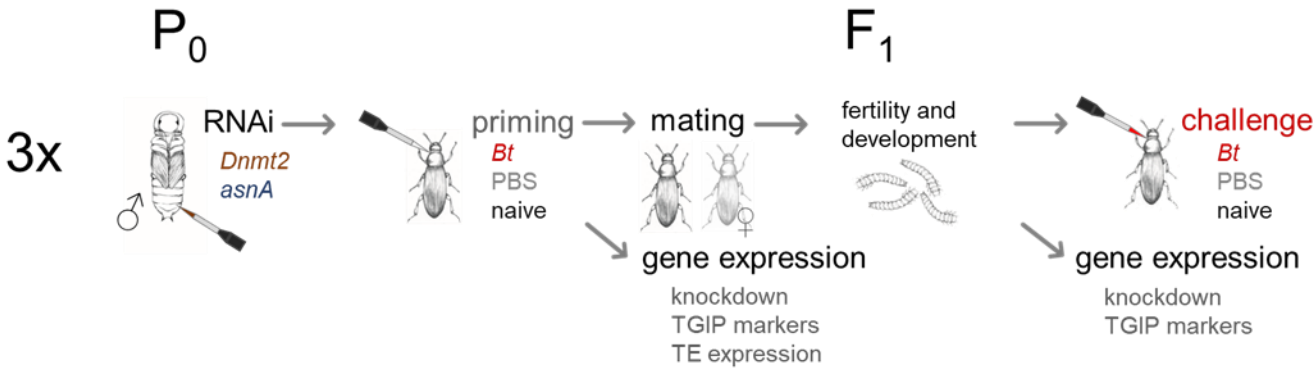
453 Tables and Figures

454 **Table 1** *Dnmt2 expression after paternal RNAi and priming* in the treated males and their adult offspring. Given
455 is the relative expression compared to RNAi control, per priming control group, for the knockdown target gene
456 *Dnmt2* normalized over the expression of two housekeeping genes. Per treatment combination, generation
457 and three experimental replicates five samples comprising of 2-5 individuals were used.

Gene	Treatment		P			F_1		
	RNAi	Priming	rel. expression	95% C.I.	p value	rel. expression	95% C.I.	p value
Dnmt2	*Dnmt2*	bacterial	**0.177**	0.03 - 1.73	**<0.001**	1.116	0.54 - 2.04	0.284
		control	**0.088**	0.04 - 0.48	**<0.001**	1.061	0.53 - 1.97	0.530
		naive	**0.112**	0.03 - 0.73	**<0.001**	0.955	0.5 - 1.76	0.620
	control	bacterial	0.897	0.27 - 5.90	0.571	1.06	0.52 - 1.94	0.519
		naive	1.175	0.26 - 7.21	0.384	1.092	0.53 - 3.63	0.480

458



459

460 **Figure 1** Overview of the experimental design combining paternal RNAi to knockdown Dnmt2 with paternal
461 TGIP. The entire experiment was conducted in three replicates (asnA=RNAi control; PBS=injection control;
462 naïve=handling control).

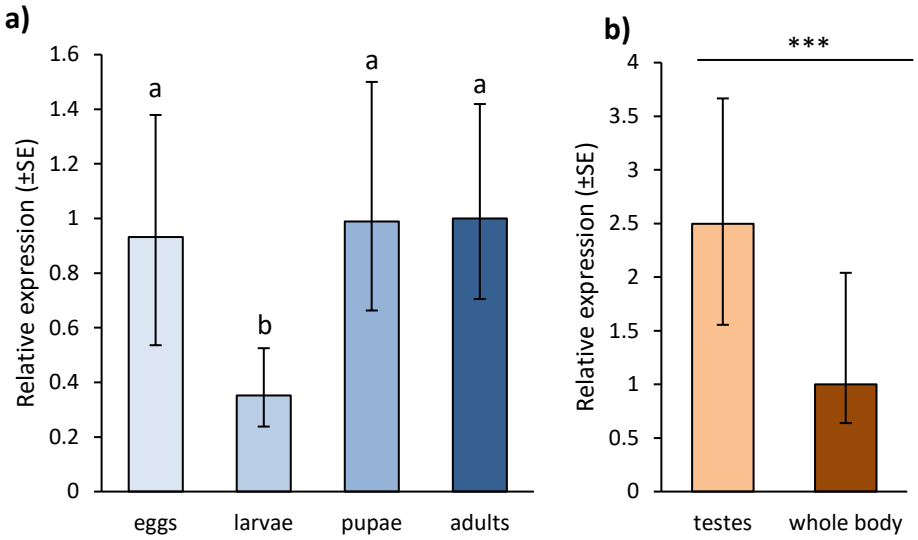


Figure 2 *Dnmt2* gene expression **a)** Relative expression in four distinct life stages of *T. castaneum* compared to adult samples (eggs: n=4 pools of 500-1000 eggs, 24h-48h post oviposition; larvae: n=7 pools of 10 larvae, 14-19 dpo; pupae: n=8 pools of 6 individuals; adults: n=8 pools of 6 individuals, one week after eclosion). **b)** Relative expression in testes and male whole-body samples (n=6). Different lettering and asterisks indicate significant differences (p≤0.001).

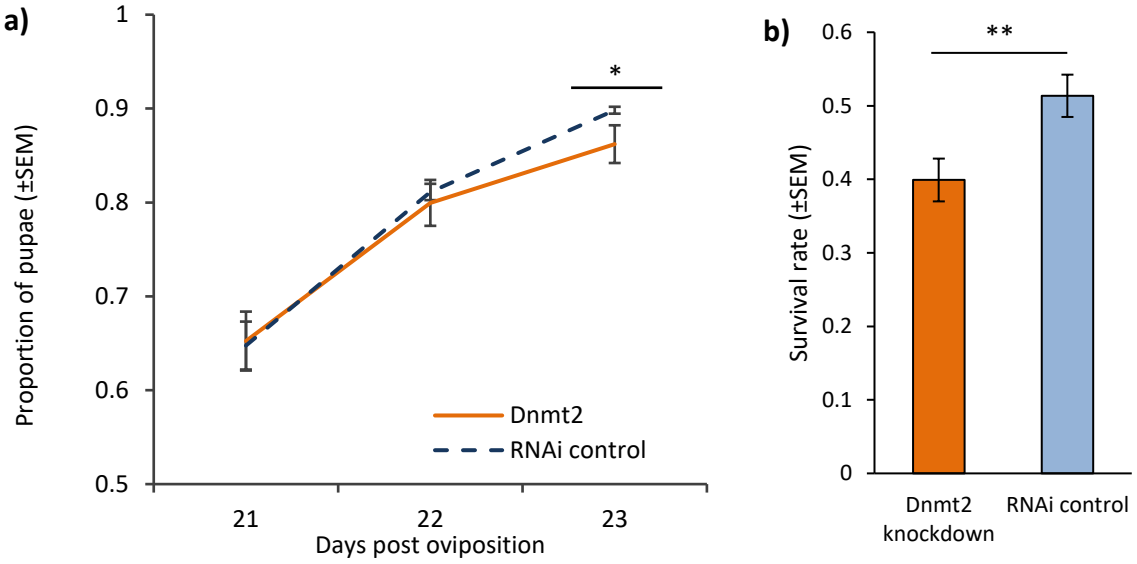


Figure 3 Effects of paternal *Dnmt2* knock-down on offspring **a)** Pupation rate of F_1 generation 21-23 dpo (±SEM for three experimental replicates) **b)** Survival of the F_1 generation after bacterial challenge according to paternal RNAi treatment. Shown are the proportions of adults that were alive four days post injection with a of *B. thuringiensis* (±SEM for three experimental replicates). Asterisks indicate significant differences (*=p<0.05, **=p<0.01).