Title: Paternal knockdown of tRNA (cytosine-5-)-methyltransferase (*Dnmt2*) increases offspring susceptibility to infection in flour beetles

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Abstract

Intergenerational effects from fathers to offspring are increasingly reported in 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 for at least two generations. Here we studied a potential role of the *Dnmt2* gene (renamed as *Trdmt1* in humans), which encodes a highly conserved enzyme that methylates specific cytosines of a set of tRNAs and has previously been reported to be involved in intergenerational 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 immune priming 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 cytosine methylation not only occurs on DNA, but also on a variety of RNAs[11]. Cytosine methylation is facilitated by a conserved family of enzymes called DNA methyltransferases (Dnmts), 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 other 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 both names remain in use[3,17]. The methylation mark provided by *Dnmt2* protects the tRNA molecule against cleavage, which can be induced by different stressors[15]. 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[18,19]. This paternal transmission is dependent on *Dnmt2,* which demonstrates the importance of this gene in non-genetic inheritance[20,21]. Furthermore, *Dnmt2* is essential to the non-Mendelian inheritance of two paramutations in mice[22]. Both epigenetic modulations - one in the Kit gene leading to changes in fur color, the other in the Sox9 gene causing overgrowth - depend on DNMT2 in order to be transmitted to the offspring generation[22].

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[23]. 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[24,25]. 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 defense. A wealth of studies in invertebrates now provide evidence for immune priming, i.e. enhanced survival upon a secondary encounter with a pathogen[26–28]. In some species it has been shown that the immune priming can also be transferred to the offspring[27,29,30]. This phenomenon is called transgenerational immune priming (TGIP)[28,31], although in most cases only a transmission to the direct offspring (F1) has been demonstrated, i.e., intergenerational immune priming would be the more correct term. While maternal transfer appears to be a relatively common phenomenon, reports about paternal immune priming via fathers are scarce[27,32]. The red flour beetle, *Tribolium castaneum* is the first example where we demonstrated paternal immune priming against a variety of bacterial pathogens[32–34]. Additionally, as paternal immune priming increased resistance also of the F2 generation[34], 'truly' trans-generational immune priming seems to occur in *T. castaneum*. However, while recent studies are beginning to shed some light on maternal immune priming[35,36], the mechanisms underlying paternal priming 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[28]. Finally, in another beetle, *Tenebrio molitor* priming of adults and larvae decreased overall RNA methylation within the generation, hinting at a possible involvement of RNA-methylating processes[37].

T. castaneum possesses two Dnmt genes: one *Dnmt1* and one *Dnmt2/Trdmt1* homolog[38]. The beetle seems to lack any functional levels of CpG DNA methylation[4,39,40], but *Dnmt1* is nevertheless expressed across all life stages and is needed for proper embryonic development[40]. 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

effects. We combined a paternal knockdown with immune priming, to investigate whether *Dnmt2* is involved in and possibly provides the epigenetic mechanism behind paternal immune priming.

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[38] and molecular tools, *e.g.* easily established systemic RNAi and genome editing[41–44] allow for genetic analyses of ecologically relevant phenomena. For this study, the *T. castaneum* line Cro 1 was used, which was established from 165 pairs of wild caught beetles collected in Croatia in June 2010[45]. Beetles were maintained in plastic breeding boxes with foam stoppers to ensure air circulation. Standard breeding conditions were 30°C and 70% humidity with a 12-hour light/dark cycle. As food source 250g of heat sterilized (75°C) organic wheat flour (type550) containing 5% brewer's yeast were given.

2.2 Gene expression of *Dnmt2*

To assess the expression of *Dnmt2* (gene ID: LOC663081, TcasGA2_TC005511) throughout the life cycle of the beetle, the four distinct life stages were sampled: eggs (n=4 pools of 500-1000 eggs, 24h-48h post oviposition), larvae (n=7 pools of 10 larvae, 14-19 days post oviposition (dpo)) pupae (n=8 pools of 6 individuals), virgin adults (n=8 pools of 6 individuals, one week after eclosion). For pupae and adults, half of the pooled samples contained females and the other half males in order to test also for differential expression between the sexes. Furthermore, gonads were dissected from unmated adult males. All samples were shock frozen in liquid nitrogen. Total RNA was extracted, and genomic DNA digested by combining Trizol (Ambion RNA by Life Technologies GmbH, Darmstadt, Germany) and chloroform treatment with the use of the Total RNA extraction kit (Promega GmbH, Mannheim, Germany) as described in Eggert *et al.*[33].

Extracted RNA was reverse transcribed to cDNA with the RevertAid First Strand cDNA kit (Thermo Fisher Scientific, Waltham, MA USA) using provided oligo-dTs. In the following RT qPCR with a Light-Cycler480 (Roche) and Kapa SYBR Fast (Kapa Biosystems, Sigma-Aldrich), each sample was

used in two technical replicates. Further analysis was conducted as described in Eggert *et al*[33]. and replicates were used in further analysis if the standard deviation between their crossing point values was below 0.5, otherwise the reaction was repeated. Previously, high primer efficiency had been confirmed and where possible it was made sure that primers crossed exon-intron boundaries (Table S1). The housekeeping genes ribosomal proteins rp49 and rpl13a were used for normalization of the expression of the target genes.

2.3 Paternal *Dnmt2* knockdown and TGIP

We aimed to downregulate *Dnmt2* through paternal RNAi and to investigate whether this knockdown 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.

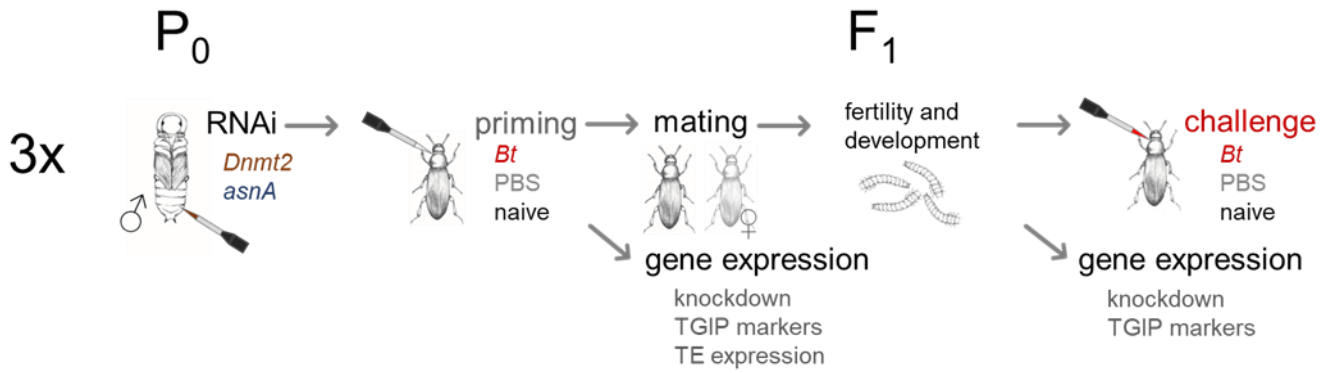


Figure 1 *Overview of the experimental design combining paternal RNAi to knockdown Dnmt2 with paternal TGIP. The entire experiment was conducted in three replicates (asnA=RNAi control; PBS=injection control; naïve=handling control).*

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[38] (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)[43]. The *Dnmt2* dsRNA construct has been previously used in the ibeetle RNAi scan[42] (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[32,33]. Bacterial cultures were grown overnight as previously described[46]. 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

158 and stress-related genes (*hsp83*, *nimB* and *PGRP*; Table S1) was analyzed, based on a previous study
159 showing effects of paternal priming on gene expression[33]. For each RNAi*priming treatment
160 combination and block five samples were taken consisting of a pool of two to five individuals. RNA
161 extraction, cDNA reverse transcription and RT qPCR were performed as described above (2.2).
162 Finally, we also analyzed the expression of seven transposable elements (TEs) (Table S1), because the
163 absence of *Dnmt2* can cause the activation of TEs[23]. Because of the lack of polyadenylation of some
164 TE transcripts, we used random hexamer primers for cDNA reverse transcription in this case (Thermo
165 Fisher Scientific, Waltham, MA USA).

166 2.3.4 Production and development of offspring generation

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

174 2.3.5 Gene expression in the offspring generation

175 One week after the majority of the offspring generation had eclosed, five pools per RNAi and priming
176 treatment combination and experimental replicate were sampled for gene expression analysis. Each
177 sample consisted of five adult beetles of unknown sex. To avoid pseudo-replication only one beetle
178 per family was used. Again, the expression of *Dnmt2* and three potential TGIP marker genes[33] (*hsp83*,
179 *nimB*, *PGRP*) was analyzed as described above (2.2).

180 2.3.6 Bacterial challenge of adult offspring

181 One week after their eclosion, adults of the F1 offspring generation were submitted to a potentially
182 lethal bacterial injection (challenge). For this, bacteria from the same *B. thuringiensis* stock as for the
183 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 Relative Expression Software Tool (REST2009 software)[47] [47]as described in Eggert *et al.*[33] In short, this software enables comparisons between treatment and control groups of up to 16 samples, while using the ΔΔ Ct method, normalizing expression via two housekeeping genes and taking primer efficiencies into account. REST2009 uses a Pair Wise Fixed Reallocation Randomization Test to determine significant differences[47,48].

All other analyses were performed in RStudio version 0.99.467[49] under R version 3.3.3[50] using additional packages lme4[51] and MASS[52]. 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.

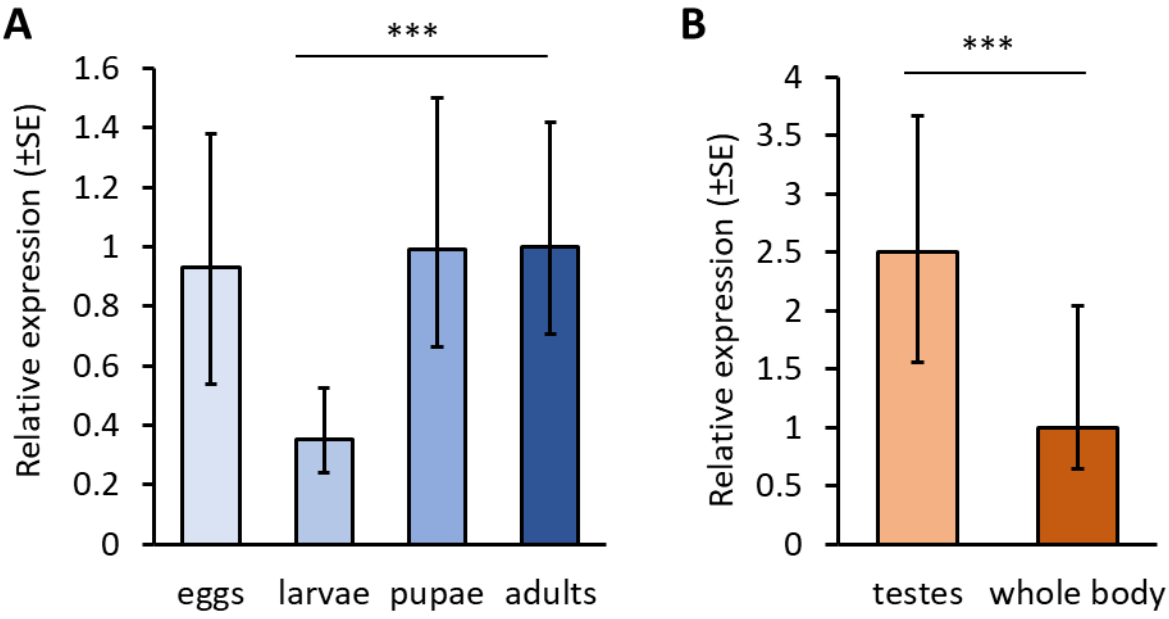
3 Results

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 **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). Asterisks indicate significant differences (p<0.001) according to statistical analysis using Pair Wise Fixed Reallocation Randomization Test (REST2009 software)[47].

3.3 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).

3.3.1 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).

3.3.2 Knockdown of *Dnmt2*

We confirmed the knockdown of *Dnmt2* after pupal RNAi in a subgroup of the adult, one day after the priming procedure, i.e., 13 days after knockdown. *Dnmt2* was significantly downregulated (to between 8.8% and 17.7%) compared to RNAi control regardless of the received priming treatment (Figure 3). As expected, *Dnmt2* mRNA had returned to normal levels in the adult offspring and there were no significant differences between the RNAi treatments (Figure 3). Additionally, the paternal priming procedure did not affect *Dnmt2* expression in the adult offspring (Figure 3).

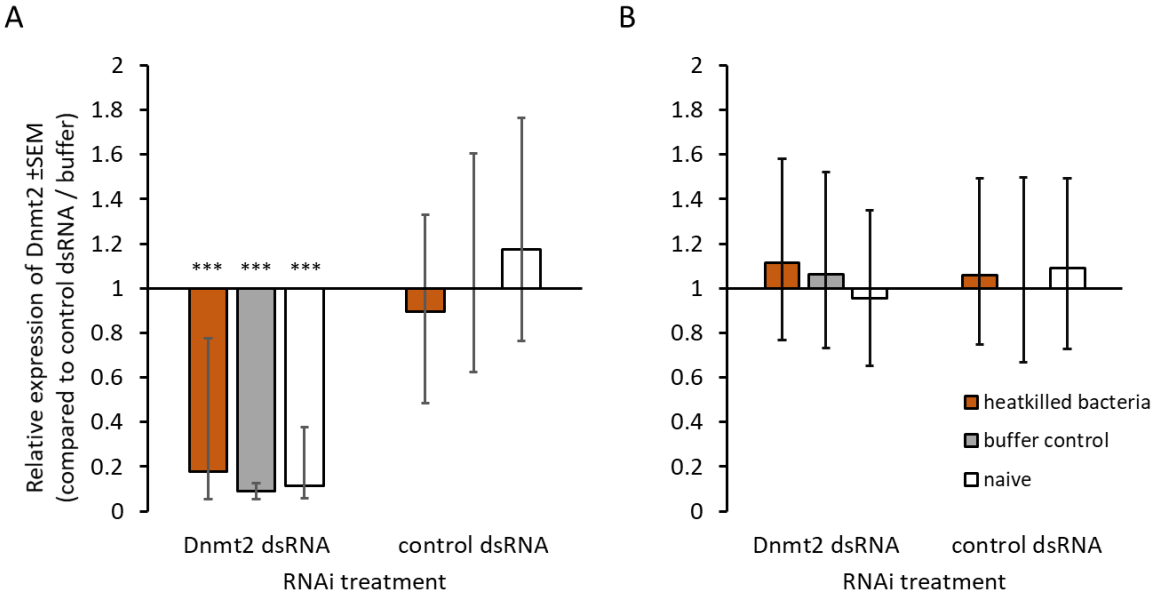


Figure 3 Knockdown of Dnmt2. A) Relative Dnmt2 expression in males of the parental generation for the three priming treatments (heat killed bacteria, naïve and buffer control) and two RNAi treatments (Dnmt2 dsRNA and control dsRNA). Each group was compared with the control dsRNA/ buffer control (n=12-14). B) Relative Dnmt2 expression in adults of the F1 generation for the three paternal priming treatments (heat

killed bacteria, naïve and buffer control) and two RNAi treatments (Dnmt2 dsRNA and control dsRNA). Each group was compared with the control dsRNA/ buffer control (n=12-15). Asterisks indicate significant differences ($p<0.001$) according to statistical analysis using Pair Wise Fixed Reallocation Randomization Test (REST2009 software)[47].

3.3.3 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).

3.3.4 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 4A, 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 4A). 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).

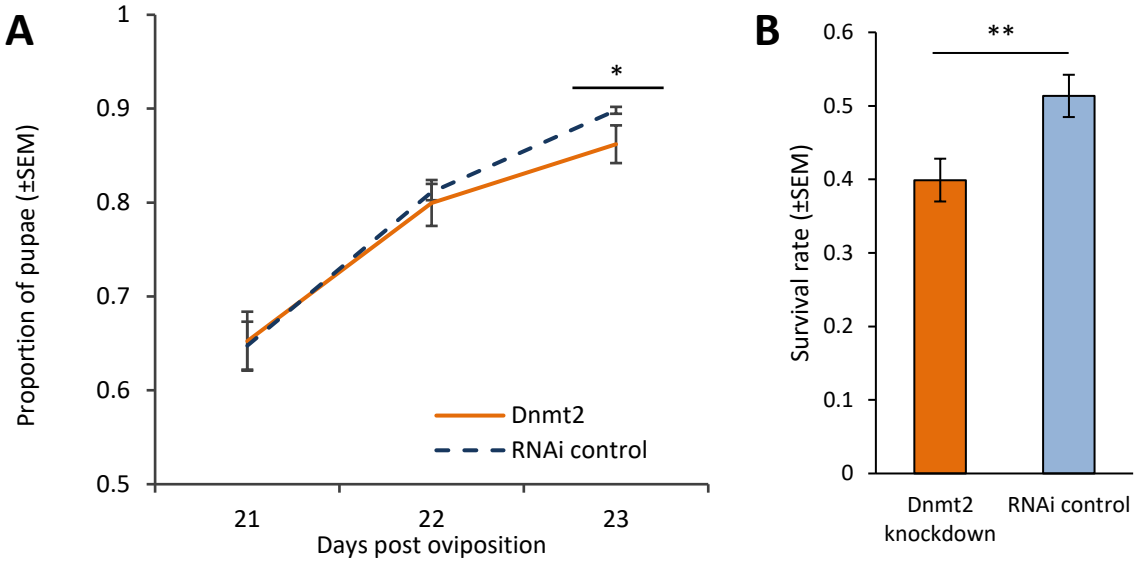


Figure 3 Effects of paternal *Dnmt2* knockdown on offspring. **A)** Pupation rate of F_1 generation 21-23 dpo (±SEM for three experimental replicates) **B)** Survival of the F_1 generation after bacterial challenge according

to paternal RNAi treatment. Shown are the proportions of adults that were alive four days post injection with *B. thuringiensis* (±SEM for three experimental replicates). Asterisks indicate significant differences (*=p<0.05, **=p<0.01).

3.3.5 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[33]. 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*[23]. 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).

3.3.6 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 4B), 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

297 *Dnmt2* can be found in almost all eukaryote species and is the most conserved member of the Dnmt
298 family[53]. It also has a function in some organisms lacking one or both other Dnmts and which are often
299 devoid of any functional DNA methylation system[13]. This also appears to be the case in *T. castaneum*,
300 which lacks *Dnmt3* and does not have any functional CpG DNA methylation, but still expresses
301 *Dnmt2*[4,38–40]. Although, we know today that *Dnmt2* methylates tRNA and not DNA, it remains unclear
302 what the role of this epigenetic mechanism is. We observed that *Dnmt2* mRNA transcripts are present
303 at relatively low but consistent levels in all life stages and in both sexes of the beetle, therefore the
304 enzyme likely functions in both males and females and throughout the entire life cycle. In fruit flies,
305 mice and humans, *Dnmt2* or its mammalian ortholog *Trdmt1* canonically methylate a small set of
306 tRNAs[14,15,54], which are highly abundant in sperm[55] and have been shown to be involved in paternal
307 transmission of metabolic phenotypes in mice[18,19]. The significantly higher expression of *Dnmt2* that
308 we observed in testes of *T. castaneum* could hint at the involvement of *Dnmt2* in paternal epigenetic
309 inheritance also in this beetle.

310 We used systemic paternal RNAi, to investigate how a *Dnmt2* knockdown would affect offspring
311 phenotypes and to discover a potential role of this gene in intergenerational epigenetic inheritance,
312 specifically from father to offspring. The offspring of *Dnmt2* RNAi-treated fathers needed longer to
313 reach pupation and thus remained longer in the more vulnerable larval stage[34]. They also died at a
314 higher rate from a *B. thuringiensis* infection, which may point towards a generally higher stress
315 sensitivity. In recent years, it has become clear that biological functions of *Dnmt2* are more easily
316 detected under stress conditions[13]. Increased sensitivity to thermal and oxidative stress has been
317 observed in *D. melanogaster Dnmt2* mutants[15], while overexpression of the same gene led to increased
318 stress tolerance[56]. During the stress response, *Dnmt2* appears to control the fragmentation of tRNA
319 and can be located at cellular stress compartments[15,57]. More recently, it has also been demonstrated
320 that the knockout of *Dnmt2* leads to a decline of immune function with ageing in adult flies[58]. Finally,
321 its absence disrupts the small interfering RNA pathway by inhibiting dsRNA degradation by *Dicer*[57].
322 However, all these effects occur in the treated generation and have not been tested for an offspring
323 generation. In our case the developmental and immunological effects were observed in the offspring
324 of treated fathers, even though offspring themselves exhibited normal *Dnmt2* expression, which

suggests intergenerational effects. A role in intergenerational 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[20]. Therefore, further studies are needed to clarify the role that *Dnmt2* plays in enabling appropriate stress responses within and across generations.

We combined the knockdown of *Dnmt2* with a paternal priming treatment, to determine whether the activity of the tRNA (cytosine-5-)-methyltransferase affects the transfer of priming information from father to offspring. We did not observe any paternal immune priming in the present study, as offspring survival of bacterial infection was independent of the paternal priming treatment. Furthermore, we did not observe any upregulation in the previously described marker genes for immune and stress responses[33] nor any changes in the expression of transposable elements. This surprising lack of any priming effect (in contrast to previous studies)[32–34] might have been caused by the wounding associated with the injection of dsRNA into all fathers for the pupal RNAi treatment, which took place before the priming treatment (note that we included an RNAi control, but no fully naïve control for the RNAi treatment, Fig. 1). As wounding activates immune responses[59], this could blur the differences between primed and non-primed fathers.[32,60]. Alternatively, the pupal RNAi injections might have inhibited priming. Lastly, although parental priming repeatable in *T. castaneum* under normal conditions [32,33,60], it is not observed in every experiment[60] and all beetle populations[61].

We did not observe any effects of the Dnmt2 knockdown on expression of the studied TEs, in contrast to *D. melanogaster Dnmt2* mutants that showed increased TE expression[23]. However, further studies are needed to make any firm conclusions, because the lack of expression differences for a limited set of TEs does not exclude the possibility that *Dnmt2* plays a role in the regulation of TEs in *T. castaneum*.

In plants, flies and mice the absence of *Dnmt2*/*Trdmt1* is not lethal under standard conditions and mutants remain fertile[14]. The same appears to be true in the case of *T. castaneum*, where we did not observe any mortality nor apparent phenotypic changes after a significant downregulation of *Dnmt2*. Additionally, male fertility was not affected by the knockdown under *ad libitum* conditions. Therefore, the maintenance of knockout lines appears feasible, which makes this gene a suitable target for 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 related to *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 for 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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Supplementary materials

Table S 1 Primer sequences and efficiencies for RTqpCR

Table S2 Relative expression of TGIP marker genes in parental (P_0) and offspring (F_1) generation compared to expression in RNAi control*priming control group (normalized over two housekeeping genes (n=15). Statistical analysis was performed using Pair Wise Fixed Reallocation Randomization Test (REST2009 software)[1]

Table S3 Relative expression of TEs after RNAi and priming treatment in parental (P_0) generation (expression relative to two housekeeping genes and RNAi control* naïve group, n=6-8). Statistical analysis was performed using Pair Wise Fixed Reallocation Randomization Test (REST2009 software)[1]

Figure S1 Survival of RNAi treatment **a)** Survival rates of mature adult males seven days post eclosion (n= 8-11 pools of 13-20 individuals per RNAi treatment and replicate). **b)** Survival rates of mature adults eight days post eclosion and 24 h post priming injection (± SEM for three experimental replicates, n=25-70 per RNAi treatment, priming treatment and replicate). Different letters indicate significant differences.

Figure S2 Male fertility after pupal RNAi treatment and adult priming injections. Shown is the offspring number from a 24h mating/oviposition period for three replicates (± SEM) consisting of 12-50 mating pairs each.

Figure S3 Development of offspring after paternal priming with *B. thuringiensis* **a)** pupation rate (±SEM for three experimental replicates) **b)** proportion of eclosed adults (±SEM for three experimental replicates) *on 26 dpo.*

Figure S4 Survival of F_1 generation after bacterial challenge according to paternal priming treatment. Shown are the proportions of adult offspring that were alive four days post injection with live *B. thuringiensis* for three independent experimental replicates.