Paternal knockdown of *Dnmt2* increases offspring susceptibility to bacterial infection

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Abstract

CpG Methylation of polynucleotides is one of the most studied epigenetic mechanisms that enable organisms to change their phenotype without altering the genotype. More recently CpG methylation occurring on small noncoding RNAs, especially of certain transfer RNAs has come into focus. This modification is established by the most conserved member of the DNA methyltransferase family, *Dnmt2*.

Dnmt2 has been indicated in transferring paternal phenotypes to offspring in mice and its absence leads to an increased sensitivity to a variety of stressors in *Drosophila melanogaster*. We therefore hypothesise that it also might play a role in paternal transgenerational immune priming, which can be observed in the red flour beetle *Tribolium castaneum*, where exposure to a non-lethal dose of bacteria in fathers protects their offspring against a potentially lethal dose of the same pathogen.

We were able to show that *Dnmt2* is expressed throughout the entire life cycle of the beetle and that expression is significantly higher in the testes. We then combined a knockdown of *Dnmt2* via pupal RNAi with a bacterial priming treatment in the eclosed adults and monitored the effects on their offspring. We used the entomopathogenic bacterium *Bacillus thuringiensis* for priming and challenge injections in adult fathers and offspring respectively.

In the paternal generation, neither viability nor fertility were affected by either RNAi or priming treatment compared to the respective controls. *Dnmt2* RNAi treatment led to a significant

downregulation and slowed down the development in the offspring larvae. Although, we could not observe a significant paternal priming effect independent of treatment, paternal knockdown led to increased mortality after bacterial injection with *B. thuringiensis*.

This demonstrates again an increased stress sensitivity caused by a lack of *Dnmt2*. Furthermore, to the best of our knowledge this is the first instance where this effect was observed in the offspring generation. In conclusion, our results highlight the importance of *Dnmt2* and show the need to further investigate this enzyme and its function in tRNA methylation and paternal non-genetic inheritance.

1 Introduction

Phenotypic plasticity is enabled by epigenetic mechanisms such as the methylation of polynucleotides (1,2). In insects, we can find many instances of this phenomenon, ranging from caste determination to phase polyphenism (3–7). One of these epigenetic modifications is the covalent binding of a methyl group to a cytosine followed by a guanine, *i.e.* CpG methylation (8). This not only occurs on DNA, where it has been extensively studied but also on a variety of RNAs, including small ncRNAs. The reaction is facilitated by a conserved family of enzymes called DNA methyltransferases, which are found in most but not all animals (8,9). *Dnmt2* is the most evolutionary 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 (10). While *Dnmt1* and *Dnmt3* are responsible for methylation of DNA, *Dnmt2* is involved in modifying RNAs, especially tRNAs (11–13). The methylation mark on certain tRNA protects the molecule against cleavage, which can be induced by different stressors (12). 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 (14,15). Furthermore, this paternal transmission is dependent on *Dnmt2* which demonstrates the importance of the enzyme in non-genetic inheritance (16).

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 herbicide treatment caused higher mortality compared to wildtype and control flies (12). Furthermore, heat shock treatment of flies lacking *Dnmt2* led to the accumulation of transposable elements and changed gene expression (17). 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 (18,19). It has been therefore proposed that the enzyme is involved in adaptive immunity and aid in defending against or adapting to pathogens (10).

A wealth of studies on invertebrates has shown so-called immune priming, an increased survival rate upon a secondary encounter with a pathogen, which can be considered a phenotypic plastic trait that enables the individual to adapt instantaneously to a changed environment (20–22). In some species it

has been shown that the immune priming can also be transferred to the offspring (21,23,24). While maternal transfer appears to be a relatively common phenomenon, reports about paternal transgenerational immune priming (TGIP) are scarce (21,25). The red flour beetle, *Tribolium castaneum* is one example where paternal TGIP against a variety of bacterial pathogens has been demonstrated (25–27). However, the mechanisms underlying TGIP remain elusive. But the paternal route of priming narrows down the possibilities by which the information could be transferred, due to the limited contact between father and sired offspring and thereby makes the involvement of epigenetic modifications, especially methylation of sperm RNA more likely (22). 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* (28).

T. castaneum possesses two sequences encoding for DNMTs, one *Dnmt1* and one *Dnmt2* homolog (29). Although, the beetle seems to lack any functional levels of CpG DNA methylation (30,31), *Dnmt1* is nevertheless expressed across all life stages and is needed for proper embryonic development (submitted). But 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 further investigate this enzyme. Finally, we combined a knockdown with paternal TGIP, to investigate whether *Dnmt2* is involved in and possibly provides the epigenetic mechanism behind this phenomenon.

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. Its status is aided by the availability of a fully sequenced genome (29) and modern molecular tools, *e.g.* RNAi (32–34). For this study a *T. castaneum* line was used, which was established from about 200 wild caught beetles collected in Croatia in June 2010 (35). 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 sterilised (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* throughout the life cycle of the beetle, the four distinct life stages were sampled (eggs (n=4 pools of 100-200µl, 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.* (26).

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.* (26) 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 normalisation 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. For this, around 2000 one-week old adult beetles 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 more, independent beetle populations on the two following days, producing three experimental replicates.

2.3.1 Paternal RNAi

Upon reaching the pupal stage, the sex of the beetles was determined, and male pupae were prepped for RNAi treatment, while females were individualised 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 immobilise 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) (34). 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 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 was recorded daily from three to six days post injection.

2.3.2 TGIP

When it was certain that all surviving beetles from the RNAi treatment had reached sexual maturity seven days after eclosion, they were injected with heat killed bacteria to achieve a priming effect. Beetles were injected with a suspension of heat killed *B. thuringiensis* (DSM no. 2046, obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ)) containing around 37,000 cells in phosphate buffered saline (PBS). *B. thuringiensis* has been successfully used in prior TGIP experiments and is pathogenic to the beetle when introduced through septic wounding (25,26). Bacterial cultures were grown overnight as previously described (36). 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 success for *Dnmt2*. Additionally, to the expression of *Dnmt2*, the expression of three immunity or stress related genes (*hsp83*, *nimB* and *PGRP*; Table S1) was analysed, which expression can be affected in the offspring upon paternal priming (26). 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 (2.2). Finally, we also analysed the expression of seven transposable elements (Table S1), because the absence of *Dnmt2* can cause the activation of some of these (17). Because of the lack of polyadenylation on TE transcripts, we in this case used random hexamer primers for cDNA reverse transcription (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 for the F1 generation, larvae from each pair were counted and up to six individuals were individualised 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 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*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 analysed as described above (2.3.3).

2.3.6 Bacterial challenge of adult offspring

One week after their eclosion, adults of the F1 generation were submitted to a potentially lethal bacterial injection (challenge). For this challenge, 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 the same as for the priming and again included an injection control and a naïve group. The dose was adjusted to around 370 bacterial cells per animal. From each family one sibling each was used for the treatment and 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 was analysed with the REST2009 software as described in *Eggert et al.* (26). All other analyses were performed in RStudio version 0.99.467 (37) under R version 3.3.3 (38) using additional packages lme4 (39) and MASS (40).

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 analysed 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 role or function of *Dnmt2* in *T. castaneum*, we monitored its expression throughout the life cycle of the beetle. We compared the expression of *Dnmt2* relative to two housekeeping genes across the four different life stages (egg, larvae, pupae and adult) of the holometabolous life cycle. The levels of *Dnmt2* transcripts 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). Although while still a detectable amount, larvae expressed significantly less *Dnmt2* than adults (relative expression=0.352, n=7, p<0.001). Additionally, *Dnmt2* appears to serve functions in both sexes 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 analysed the expression of *Dnmt2* in the reproductive tissue of the male beetles and compared it to whole body samples of the same sex. Expression in the testes could hint at an involvement of the gene 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), suggest a possible relevance of the protein in male reproduction.

3.2 Paternal *Dnmt2* knockdown and TGIP

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

3.2.1 Survival of RNAi and priming injections

The RNAi treatment with *Dnmt2* dsRNA did not increase mortality or hinder the eclosion of the treated pupae (Figure S1). Injections of male pupae did not significantly alter survival rates neither following the RNAi (GLMM, df=1, X^2=0.16, p=0.69) nor the priming treatment in the mature adults ten days later (GLMM, df=1, X^2=0.04, p=0.84). However, the priming procedure itself led to significantly increased mortality regardless 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 1).

3.2.2 Successful knockdown of *Dnmt2*

One day after the priming procedure, we confirmed the successful 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). Aditionally, the paternal priming procedure did not affect *Dnmt2* expression in the adult offspring (Table 1).

3.2.3 Knockdown of *Dnmt2* and adult priming do 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=0.8).

3.2.4 Paternal knockdown but not priming affects offspring development

We monitored offspring development by measuring the proportion of pupae over three consecutive days and the proportion of eclosed adults 26 dpo. Animals from all six treatment combinations (RNAi*priming) showed similar pupation rates 21 and 22 dpo (Figure 2, Figure S2). But 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 2, Figure S2). The proportion of eclosed adults 26 dpo was not significantly affected by any paternal treatment (Figure 2; Figure S2).

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). Also, none of the paternal treatments (RNAi*priming) did affect the expression of *Dnmt2* in the adult offspring (Table 1).

For the same animals from the paternal generation we also measured the expression of seven TEs. Genenncher *et al.* (17) observed that the absence of *Dnmt2* and the exposure to heat stress lead 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 the exposure to a wounding stress (priming injection) (Table S3).

3.2.6 Paternal Dnmt2 knockdown reduces 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 S3), which possibly can be explained by the additional wounding all fathers received during the RNAi 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=0.0053, Figure 3), demonstrating that *Dnmt2* impacts stress sensitivity and that its reduction can increase susceptibility towards pathogens.

4 Discussion

Dnmt2 can be found in almost every species and is the most conserved member of the Dnmt family (41). It has also a function in some organisms lacking all other Dnmts and a functional DNA methylation system (10). This also appears to be the case in *T. castaneum*, which has an incomplete set of Dnmts and no functional DNA methylation (29–31), but still expresses *Dnmt2*. We observed that *Dnmt2* mRNA transcripts are present in all life stages and in similar levels in both sexes of the beetle, therefore the enzyme might have a sex-independent role throughout the entire life cycle. *Dnmt2* exclusively methylates a small set of tRNAs (8), which are highly abundant in sperm (42) and have been shown to be involved in paternal transmission of phenotypes in mice (14,15). The significantly higher expression in *T. castaneum* testes indicates the possibility that this might also be a major function of *Dnmt2* in the beetle.

We combined the knockdown of *Dnmt2* with a TGIP treatment, to determine whether this enzyme is involved in the transfer of the information from father to offspring. We did not observe a TGIP effect in this study. Offspring survival did not depend on paternal priming treatment. Furthermore, we did not observe an upregulation in certain marker immune and stress response genes as has been previously described for paternal TGIP in *T. castaneum* (26) nor did TEs increase in abundance as observed in *D. melanogaster Dnmt2* mutants (17). The absence of TGIP in this case might be caused by the wounding of the animals during pupal RNAi treatment. To our knowledge there is no data on how injuries sustained during the pupal phase might influence later responses. But, in a few experiments wounding during control treatment also increased survival of a later bacterial challenge (25,43). Therefore, a potential wounding effect might have masked the survival benefits of TGIP. On the other hand, the pupal RNAi injections could possibly also inhibit any later priming. Lastly, although TGIP in *T. castaneum* is robust and repeatable (25,26,43), it also has become apparent that this phenomenon cannot be observed in every experiment (43) nor beetle population (44).

In plants, flies and mice the absence of *Dnmt2* is not lethal under standard conditions and mutants remain fertile (11). The same appears to be true in the case of *T. castaneum*, where we did not observe any additional mortality nor apparent phenotypic changes after a significant downregulation of *Dnmt2*. Additionally, male fertility was not affected by the knockdown under *ad libitum* condition. Therefore, at least at first sight *Dnmt2* does not seem to fulfil an essential function in the beetle and 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.

In our experiment the offspring of *Dnmt2* RNAi treated fathers developed more slowly and exhibited a higher stress sensitivity. They took longer to reach pupation, were less well equipped to deal with a *B. thuringiensis* infection and died at a significantly higher rate than the offspring of the RNAi control. This was independent of the paternal priming treatment. In recent years, it has become clear that biological functions of *Dnmt2* are more easily detected under stress conditions (10). Increased sensitivity to thermal and oxidative stress has been observed in *D. melanogaster Dnmt2* mutants (12), while overexpression of the same gene has led to increased stress tolerance (45). During the stress response, *Dnmt2* appears to control for the fragmentation of tRNA and can be located at cellular stress compartments (12,46). Finally, its absence disrupts the small interfering RNA pathway by inhibiting dsRNA degradation by *Dicer* (46). However, the increased stress sensitivity to bacterial infection we observed here occurred in the offspring generation, which exhibited normal *Dnmt2* expression. It remains unclear if the same mechanisms are involved in this transgenerational effect. Therefore, further studies are needed to investigate more directly the effects *Dnmt2* has on tRNA methylation in *T. castaneum* and other insects besides *D. melanogaster*. Nevertheless, we here demonstrated for the first time in an invertebrate that paternal *Dnmt2* levels affect offspring phenotype, giving a new scope for non-genetic inheritance of a phenotype.

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6 Contributions

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

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8 Competing Interest Statement

The authors declare no competing interests.

9 Tables and figures

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

Gene	Treatment		P_0			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	0.53
		naive	**0.112**	0.03 - 0.73	**<0.001**	0.955	0.5 - 1.76	0.62
	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	0.48

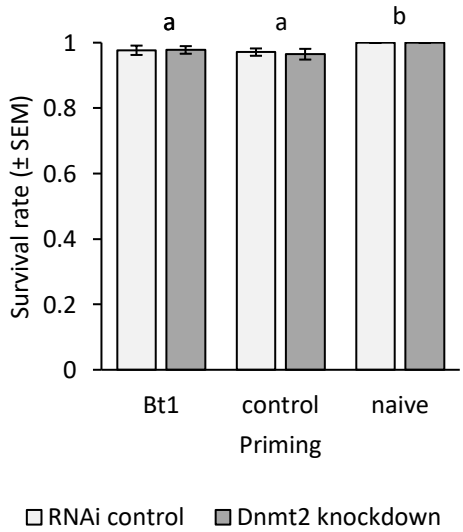

Figure 1 Survival of priming procedure according to RNAi and priming treatment 24 h post injections (± SEM for three experimental replicates, N=950). Different letters indicate significant differences.

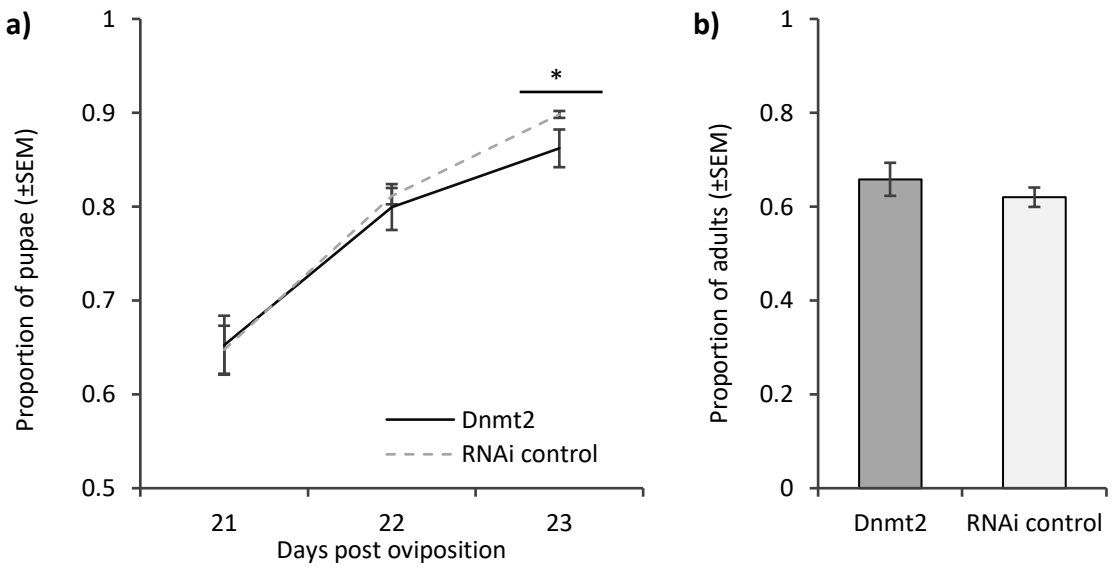

Figure 2 Development of offspring after paternal RNAi **a)** pupation rate (±SEM for three experimental replicates) **b)** proportion of eclosed adults (±SEM for three experimental replicates) on 26 dpo. Asterisk indicates significant differences.

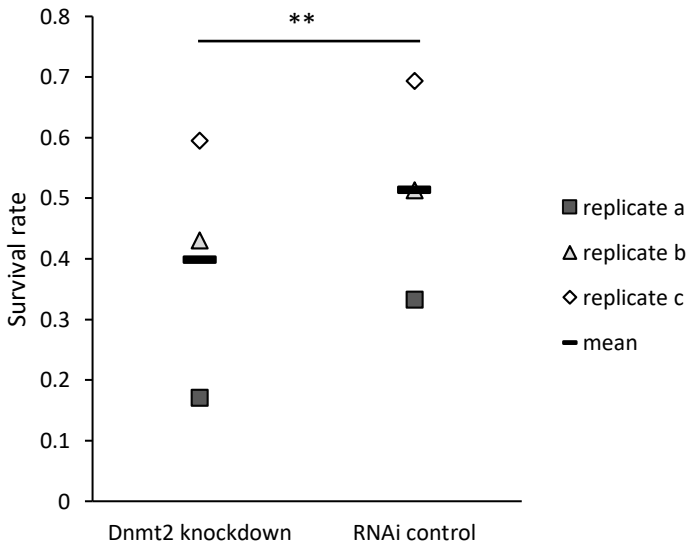

Figure 3 Survival of F1 generation after bacterial challenge according to paternal RNAi treatment. Shown are the proportions of adults that were alive four days post injection with a potentially lethal dose of *B. thuringensis*.