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1 # Transgenerational developmental effects of immune priming in the red
2 # flour beetle *Tribolium castaneum*

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14 **Abstract**

15 Immune priming, the increased chance to survive a secondary encounter with a pathogen, has been
16 described for many invertebrate species, which lack the classical adaptive immune system of
17 vertebrates. Priming can be specific even for closely related bacterial strains, last up to the entire
18 lifespan of an individual, and in some species, it can also be transferred to the offspring and is then
19 called transgenerational immune priming (TGIP). In the red flour beetle *Tribolium castaneum,* a pest
20 of stored grains, TGIP has even been shown to be transferred paternally after injection of adult
21 beetles with heat-killed *Bacillus thuringiensis*.

22 Here we studied whether TGIP in *T. castaneum* is also transferred to the second filial generation,
23 whether it can also occur after oral and injection priming of larvae and whether it has effects on
24 offspring development. We found that paternal priming with *B. thuringiensis* does not only protect
25 the first but also the second offspring generation. Also, fitness costs of the immune priming became
26 apparent, when the first filial generation produced fewer offspring. Furthermore, we used two
27 different routes of exposure to prime larvae, either by injecting them with heat-killed bacteria or
28 orally feeding them *B. thuringiensis* spore culture supernatant. Neither of the parental larval priming
29 methods led to any direct benefits regarding offspring resistance. However, the injections slowed
30 down development of the injected individuals, while oral priming with both a pathogenic and a non-
31 pathogenic strain of *B. thuringiensis* delayed offspring development.

32 The long-lasting transgenerational nature of immune priming and its impact on offspring
33 development indicate that potentially underlying epigenetic modifications might be stable over
34 several generations. Therefore, this form of phenotypic plasticity might impact pest control and
35 should be considered when using products of bacterial origin against insects.

36 ## 1 Introduction

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37 Over the last decade a wealth of new evidence has been put forward to demonstrate that invertebrate
38 immune systems can possess forms of immune memory and are sometimes capable of highly specific
39 responses (Cooper and Eleftherianos 2017; Milutinović and Kurtz 2016; Contreras-Garduño et al.
40 2016). The phenomenon enabling a stronger and faster immune response upon secondary infection
41 has been termed immune priming and shows parallels in memory and specificity to trained immunity
42 of vertebrates (Little and Kraaijeveld 2004; Kurtz and Armitage 2017; Kurtz 2005; Netea et al. 2011;
43 Melillo et al. 2018). The trigger, specificity and duration of the priming can be extremely diverse. It
44 has been shown that immune priming can be successful against bacteria (Roth et al. 2009), fungi
45 (Fisher and Hajek 2015; Gálvez and Chapuisat 2014) and viruses (Tidbury, Pedersen, and Boots
46 2011). Immune priming can be achieved by introducing a sublethal dose of the parasite, an
47 incapacitated, *e.g.* heat killed agent or using only specific molecules from the original pathogen, *e.g.*
48 lipopolysaccharides (Milutinović and Kurtz 2016; Contreras-Garduño et al. 2016). Also, the route
49 how the elicitor is introduced can vary, similar to differences in the route of infection in nature. For
50 experiments involving priming, the priming agent is most commonly introduced via septic wounding
51 and deposition into the haemocoel or orally via feeding (Milutinović and Kurtz 2016). Furthermore,
52 also abiotic factors, e.g. thermal exposure have been shown to prompt this phenomenon (Wojda and
53 Taszłow 2013; Eggert et al. 2015). Immune priming can be cross-reactive in some cases (Moret and
54 Siva-Jothy 2003), while in others the host's primed immune response can differentiate between
55 bacterial species or even strains, mounting the best protection when the same pathogen is
56 encountered twice (Roth et al. 2009; Medina Gomez et al. 2018).

57 Additionally, the duration of immune priming effects differs dramatically. In some cases, protection
58 lasts across different life stages and throughout the entire life span of an individual (Thomas and
59 Rudolf 2010; Khan et al. 2016; Pham et al. 2007). In some cases, the immune priming is even
60 transferred to the offspring generation (Milutinović and Kurtz 2016; Roth et al. 2018; Dhinaut et al.
61 2018). This transgenerational immune priming (TGIP) can occur through either parent. While for the
62 maternal side, the direct transfer of bacterial particles bound to egg-yolk protein vitellogenin has been
63 shown to be involved in certain systems (Salmela et al. 2015), the detailed mechanistic underpinnings
64 of immune priming in general and paternal TGIP in particular still remain to be discovered
65 (Milutinović et al. 2016). It has been considered that epigenetic mechanisms, including DNA
66 methylation, histone acetylation and miRNAs are involved (Vilcinskas 2016; Eggert et al. 2014).

67 As with any other immune response also the fitness costs of immune priming including those for
68 storing the information have to be considered. These costs are not constraint to a direct reduction in
69 fertility but can also become visible in delayed development or smaller body mass if the priming
70 occurs before the organism reaches maturity. Furthermore, negative effects might only become
71 visible in the offspring generation. In the Coleopteran, *Tenebrio molitor*, maternal priming prolonged
72 offspring larval development (Zanchi et al. 2011) and the strength of this effect depended on the
73 Gram type of the bacteria used for priming (Dhinaut et al. 2018). Immune priming beneficial to the
74 mother can even increase offspring susceptibility to the same parasite (Vantaux et al. 2014). These
75 are all factors demonstrating the complexity of immune priming and showing that this term probably
76 covers several distinct (Pradeu and Du Pasquier 2018). It makes predicting host-parasite co-evolution
77 and the emergence of resistance against bacterial pesticides much more difficult if we consider that
78 several forms of immune priming can occur in the same species across different life stages and
79 generations with different consequences.

80 Immune priming has intensively been studied in the red flour beetle *Tribolium castaneum*, which is a
81 widely abundant pest of stored grains. In this beetle, immune priming has been demonstrated in
82 different life stages, i.e. larvae and adults, as well as within and across generations (Milutinović et al.

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83 2016). In this species, immune priming can be highly specific, down to the bacterial strain and can be
84 passed on via both parents (Roth et al. 2009; Roth et al. 2010). Two different routes of priming and
85 infection were used. Oral infections with spores only work in larvae and the protective benefits of
86 priming with the supernatant of the spore culture have so far only been studied within generation,
87 mostly even within life stage (Milutinović et al. 2014; Futo et al. 2017; Greenwood et al. 2017).
88 Therefore, the effectiveness of the priming was only confirmed for a few days after exposure. The
89 other priming and infection method uses vegetative cells, which are inactivated for the priming and
90 are directly introduced into the body cavity via septic wounding (Tate et al. 2017; Khan et al. 2016;
91 Milutinović et al. 2016). In this case, immune priming of adults can be transferred to their offspring
92 and a protection against infection can still be observed in the adults of the offspring generation (Roth
93 et al. 2010; Eggert et al. 2014). But, these different priming techniques and routes of infection lead to
94 different responses as is evident in differential gene expression and immune system activity (Behrens
95 et al. 2014). The pathogen used in most studies of priming in *T. castaneum* is the entomopathogenic
96 and endospore forming bacterium *Bacillus thuringiensis* (Jurat-Fuentes and Jackson 2012). Proteins
97 from *B. thuringiensis*, so-called Cry toxins are widely used for their insecticidal activity in transgenic
98 crops (Lacey et al. 2015; Pardo-López et al. 2013). Therefore, the study of immune priming in this
99 host parasite model system does not only advance basic research and our understanding of the
100 invertebrate immune system but is also helpful for applied approaches and improving insect control
101 strategies.

102 With our study we shed further light on the different forms of immune priming against
103 *B. thuringiensis* that can be observed in *T. castaneum*. We here investigated the transgenerational
104 effects caused by three different types of priming, *i.e*. priming by injection of larvae and male adults
105 and oral priming of larvae by monitoring the development, fitness and survival of bacterial infection
106 (challenge). As paternal TGIP so far has only been tested in the first offspring generation (Roth et al.
107 2010), we here expanded the experimental time frame to include the adult grandparental generation,
108 investigating whether TGIP is a multigenerational phenomenon extended to more than one
109 subsequent generation. Studies on larval priming have been mainly focused on within generation
110 immune benefits (Milutinović et al. 2016). We therefore here wanted to investigate whether larval
111 TGIP via the oral or septic wounding infection route exists and whether the offspring is affected in a
112 different way by parental treatment.

113 2 Materials and Methods

114 2.1 Model organisms

115 Beetles were derived from a population originally collected in the wild in Croatia in 2010 (Milutinović
116 et al. 2013). Until the start of the experiment, beetles were kept in populations of more than 2,000
117 individuals in plastic boxes with heat sterilized (75 °C) organic wheat flour (type 550) enriched with 5
118 % brewer's yeast. Standard breeding conditions were set at 70 % humidity and 30 °C with a 12 h
119 light/dark cycle.

120 In all priming treatments and infections, different entomopathogenic gram-positive *B. thuringiensis*
121 strains were used. *B. thuringiensis* and its Cry toxins are widely used as insecticides and together
122 with *T. castaneum* form a well-established system to study host parasite co-evolution (Roth et al.
123 2009; Milutinović et al. 2013; Contreras et al. 2013; Pardo-López et al. 2013). For priming and
124 challenge by injection we used vegetative cells from *B. thuringiensis* (*Bt*) strain DSM 2046 (German
125 Collection of Microorganisms and Cell Cultures, DSMZ). For the treatments concerning priming and
126 infection by oral uptake, spores and supernatant from *Bt morrisoni bv. tenebrionis* spore cultures *(Btt*,

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127 Bacillus Genetic Stock Center, Ohio State University, Ohio, USA) were used. Additionally,
128 *Bt407cry⁻* (*Bt407*, kindly provided by Dr. Christina Nielsen-Leroux, Institute National de la
129 Recherche Agronomique, La Minière, 78285 Guyancourt Cedex, France) served as a negative control
130 in the oral priming experiment, as it does not produce Cry toxins and does not lead to immune
131 priming nor mortality upon ingestion (Milutinović et al. 2013; Milutinović et al. 2014).

132 2.2 TGIP in adults

133 In this experiment we wanted to investigate, whether paternal TGIP persists past the first filial (F_1)
134 generation (Roth et al. 2010; Eggert et al. 2014) and therefore provides survival advantages upon *Bt*
135 infection to the second filial (F_2) generation. Additionally, we measured the fertility of the primed
136 males and their offspring to determine potential costs of TGIP. For an overview of the experimental
137 design see Figure 1.

138 2.2.1 Priming of the parental (P_0) generation

139 To set up the P_0 generation for this experiment around 2000 beetles from a general stock population
140 were put into a plastic box containing 250 g of flour with yeast. After an oviposition period of 24 h
141 the adults were sieved off and put into a new box for a second 24 h oviposition period. When the
142 offspring had reached the pupal stage, their sex was determined, and all beetles were kept
143 individually from here on onwards.

144 For the priming injections one week after eclosion, 60 male adults were either injected with heat-
145 killed *Bt* suspended in PBS at a concentration of $1*10^9$ cells per ml, PBS only to control for the
146 wounding or left naïve. The priming suspension was directly injected into the dorsal vessel by
147 dorsally puncturing the epidermis between head and pronotum in a flat angle to minimize tissue
148 damage. Heat-killed *Bt* were produced from an overnight culture as previously described (Roth et al.
149 2009; Ferro et al. 2017). A nanoinjector (Drummond Nanoject II) was used for this procedure with
150 the injection volume set to 18.4 nl (~20,000 cells per injection in the *Bt* treatment). Survival after the
151 priming procedure was recorded 24 h later.

152 2.2.2 Mating and fitness of P_0 and F_1 generation

153 Single mating pairs with naïve, virgin females were set up (n=39-57). Mating pairs were kept in
154 plastic vials containing 6 g of flour and left to lay eggs for two consecutive three-day long
155 oviposition periods. Thirteen days after the end of the respective oviposition period, larvae were
156 counted for each pair and individualized into 96 well plates with flour. For the analysis, data from
157 both oviposition periods were combined.

158 The sex of the offspring was determined when they had reached the pupal stage. One female and one
159 male offspring from each single pair formed a new mating pair to produce the F_2 generation, leading
160 to mating of full siblings (n=29-53). Mating, oviposition and individualization of offspring larvae
161 were carried out in the same way as described for the parental generation with the exception of the
162 oviposition periods being shortened to 24 h. The fertility of F_1 pairs was recorded as live larvae 12
163 days post oviposition (dpo).

164 2.2.3 Bacterial Challenge of adults of F_1 and F_2 generation

165 The priming of adult males of *T. castaneum* with heat-killed *Bt* leads to an increased survival rate in
166 their adult offspring when infected with a potentially lethal dose of the same bacteria (Eggert et al.
167 2014). Whether this phenomenon is also transferred to subsequent generations has so far not been
168 investigated. We therefore exposed individuals of the F_1 and F_2 generation to a bacterial challenge

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169 after the P_0 generation had received a priming treatment. Bacteria were cultured, washed and their
170 concentration in PBS adjusted as for the priming procedure without the heat-killing step (2.2.1). One
171 week after eclosion animals of both sexes were injected with a volume of 18.4 nl. The injection either
172 contained *Bt* cells at a concentration 10^7 vegetative bacterial cells per ml (~200 cells per injection) in
173 PBS or only PBS as a control and was performed in the same manner as described for priming
174 (2.2.1). A second control consisted of a naïve group that received no injections. In the F_1 generation,
175 three adult siblings from each family were used, one for each challenge treatment (n=31-44). This
176 was the same for the F_2 generation, but here the challenge was performed on adults originating from
177 two consecutive ovipositions of the same families (oviposition 1: n=16-42, oviposition 2: n=24-45).
178 Injections were carried out in the same manner as for the priming treatment (2.2.1). Afterwards, the
179 beetles were kept in individual glass vials and their survival was recorded 24 h post challenge.

180 **2.3 Transgenerational effects of larval priming**

181 Within generation immune priming of *T. castaneum* larvae with *B. thuringiensis* can be achieved by
182 two different infection routes, *i.e.* oral ingestion of spore conditioned supernatant or by the
183 introduction of heat-killed vegetative cells into the hemolymph by pricking with a needle dipped in
184 bacterial solution or direct injection (Behrens et al. 2014; Ferro et al. 2017). Here, we investigated
185 whether priming via either of these infection routes affects fertility. For this, larvae from a 24 h
186 oviposition period of our stock population were exposed to one of two oral treatments, one of two
187 injection treatments or left naïve (for priming treatment details see 2.3.1 and 2.3.2). During the pupal
188 stage the sex of the individuals was determined and once they had reached sexual maturity, single
189 mating pairs were formed within each priming treatment (n=57-66). Pairs were allowed to mate and
190 produce eggs for two consecutive 24 h oviposition periods. Afterwards, the adults were sieved off
191 and offspring larvae were counted 14 dpo. For the analysis, data from both oviposition periods were
192 combined.

193 Furthermore, we wanted to know whether the oral or injection immune priming of larvae can also be
194 transferred to the F_1 generation, as has been observed in the priming of adult *T. castaneum* (Tate et
195 al. 2017; Roth et al. 2010). To answer this, the following TGIP experiments on larvae were
196 conducted applying oral and injection immune priming and challenge protocols. For an overview of
197 the experimental design see Figure 1.

198 **2.3.1 Oral priming and challenge of larvae**

199 For the culturing and sporulation of *B. thuringiensis* we followed the method given in Milutinović *et*
200 *al.* (2013). Milutinović *et al.* (2014) describe the methodology to orally prime larvae with *Bt* spore
201 supernatant. In short, for the oral priming and challenge the spore supernatant or spores are provided
202 to the beetle by mixing them with flour and PBS, pipetting the mixture into a 96 well plate and letting
203 the diet dry to form flour discs. In addition to the *Btt* treatment, *Bt407* was used as a negative control
204 in the priming and challenge procedure because the supernatant from its spore culture does not
205 provide a priming effect nor do its spores cause mortality upon ingestion (Milutinović et al. 2014;
206 Milutinović et al. 2013). As a third group a naive control was included with pure PBS to produce the
207 flour discs.

208 The P_0 generation originated from approximately 1000 beetles from our stock population ovipositing
209 for 24 h. Larvae of the P_0 generation were exposed to the priming diets 14 dpo for 24 h (n=320).
210 After the priming, a subgroup of the primed larvae was transferred onto naïve flour discs, on which
211 they remained until the oral challenge. The within generation challenge was performed to confirm
212 successful priming. The challenge took place 19 dpo, *i.e.* five days after the exposure to the priming

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213 diet, in a full factorial design. Besides the challenge diet of *Btt* spores, two controls were included
214 using either *Bt407* spores or flour discs prepared with pure PBS (n=40). The spore concentration was
215 adjusted to $5*10^9$ spores per mL. Larvae stayed on their respective flour discs for the rest of the
216 experiment. Survival after challenge was recorded daily for the next eight days.

217 As mentioned above, a subgroup of the F_1 generation was orally challenged as well. This group was
218 produced by the mating of single pairs coming from the same priming group. One individual from
219 each mating pair was used for each of the three challenge treatments (n=71-76). The challenge was
220 conducted in a similar manner as for the P_0 generation, but without the naïve control. Instead it
221 included two different spore concentrations to counteract the possibility of too high or too low
222 mortality rates. The spore concentration was set to either $1*10^{10}$ spores per ml (high dose) or $5*10^9$
223 spores per ml (low dose). Larvae were put on naïve flour discs at 14 dpo to ensure similar
224 development as in the P_0 generation and avoid early pupation, as the development in lose flour is
225 considerably faster than on flour discs. The challenge took place 19 dpo and again survival was
226 monitored for eight days.

227 To determine potential costs of the immune priming, we monitored the development of the remaining
228 individuals of the P_0 generation after priming that were not used in the challenge (*Btt* and *Bt407*:
229 n=196, naïve: n=280) and their offspring produced from the mating pairs, which came from the same
230 priming treatment (70-75). In the P_0, pupation rates were monitored between 21 dpo and 25 dpo and
231 the proportion of eclosed adults was recorded 27 dpo. The offspring larvae were individualized 14
232 dpo and kept in lose flour the entire time. They were checked for pupation between 19 dpo and 23
233 dpo and their eclosion rates were noted 28 dpo.

234 **2.3.2 Priming and challenge of larvae by bacterial injection**

235 Priming by injection of heat killed *Bt* cells took place 14 dpo. The larvae for this experiment came
236 from a 24 h oviposition of ~1000 beetles from our stock population. The procedure also included an
237 injection control in which only PBS was used and a naïve group (n=244). Heat-killed priming
238 bacteria were produced as described above (2.2.1). Priming injections had a volume of 18.4 nl and were
239 placed in a flat-angle laterally under the epidermis of the third-last segment using a nanoinjector
240 (Drummond Nanoject II). The bacterial concentration was adjusted to $1*10^9$ cells per ml (~20,000
241 cells per larvae). After the injection, larvae were kept individually in 96 well plates containing flour.

242 We performed a within generation injection challenge to confirm the success of the priming. During
243 the bacterial challenge 19 dpo, *i.e.* five days post priming a subgroup of the animals was injected
244 with 18.4 nl of either vegetative *Bt* cells at a concentration of $1*10^7$ cells per ml suspended in PBS or
245 only PBS (n=48). Challenge injections were placed in the dorsal vessel at a flat angle dorsally under the
246 epidermis of the first thoracic segment to minimize tissue damage. After the challenge injection, larvae
247 were continued to be kept individually, and their survival was checked seven days later. This challenge
248 procedure was performed on a subgroup of the F_1 generation in the same manner, which was produced
249 from single pairs within the same priming group, which produced eggs for two consecutive 24 h periods
250 (n=96). Again, survival was measured after seven days.

251 Also, for the injection priming, we wanted to test whether the treatment was costly and impacted the
252 development. We therefore checked the proportion of pupae in a subgroup of the P_0 generation
253 (n=196) 23 dpo and the proportion of eclosed adults in the F_1 generation (n=72-103) 27 dpo. The F_1
254 generation was produced from single mating pairs within a priming treatment and offspring larvae
255 were individualized 14 dpo, *i.e.* the age their parents had been primed.

256 **2.4 Statistical analysis**

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257 All statistical analyses were performed in R (R Development Core Team 2008) using RStudio
258 (RStudio Team 2015). Additional packages utilized included: MASS (Venables and Ripley 2002),
259 lme4 (Bates et al. 2015), multcomp (Hothorn et al. 2008) and survival (Therneau and Grambsch
260 2000). Data concerning larval survival and development until pupation were tested in a Cox
261 proportional hazard analysis, after it had been ensured that the assumption of hazards being
262 proportional over time had been fulfilled. When this was not the case, generalized linear mixed
263 effects models (GLMM) with a binomial distribution and experimental block as random factor were
264 applied on data for one specific time point for pupation rates. This method was also used to examine
265 eclosion rate. Tukey Honest Difference (THD) was applied *post hoc* to determine significant
266 differences between individual treatment groups, while adjusting the p-values for multiple testing.
267 X^2-tests were used to analyze survival after injection challenge in cases for which random factors did
268 not apply.

269 3 Results

270 3.1 Adult TGIP is transmitted to the F_2 generation

271 We first wanted to confirm successful TGIP in the adults of the F1 generation. Due to an unusually
272 high death rate in the beetles injected only with buffer, we did not observe significant differences in
273 mortality between the beetles exposed to bacteria (challenge) and the injection control regardless of
274 paternal priming (N=232, X^2=0.707, p=0.4; Figure 2a). Within the *Bt* challenged group, there was a
275 tendency towards effective TGIP, as we observed a trend towards increased survival in the group that
276 had received the paternal priming treatment compared to the priming injection control (N=69,
277 X^2=3.401, p=0.065; Figure 2a). We did not see any difference between the priming treatments in
278 response to challenge injection control (N=119, X^2=0.473, p=0.78; Figure 2a).

279 We then tested whether TGIP is also passed on to the successive generation. The challenge of the
280 adult F_2 generation proved to be effective, as significantly more beetles died after injection with live
281 bacteria than of those that received control injections (GLMM: df=1, X^2=23, p<0.001; Figure 2b).
282 Furthermore, offspring, whose grandfathers had received a priming injection with heat-killed bacteria
283 survived significantly better than those from the priming control group (GLMM: df=2, X^2=7.3,
284 p<0.05; THD: z=-2.492, p<0.05; Figure 2b). Survival in the naive control did not differ significantly
285 from the priming control (THD: z=-0.827, p=0.68), but there was a tendency towards higher
286 mortality compared to the offspring of primed grandfathers (THD: z=-2.090, p=0.09;Figure 2b).
287 Therefore, the previously described TGIP in *T. castaneum* is transmitted past the first offspring
288 generation at a comparable strength to the F_2 generation.

289 We investigated possible costs of TGIP by counting live offspring two weeks after mating as a measure
290 of reproductive success in the P_0 and F_1 generations. We could not observe any effect of parental
291 priming treatment on fertility for the P_0 (GLM: df=2, X^2=3.399, p=0.18; Figure 3a) nor the F_1
292 generation (GLM: df=2, X^2=7.19, p<0.05; THD: priming control z=-0.527, p=0.86; naïve z=2.014,
293 p=0.11, Figure 3b). However, the paternal priming control treatment significantly reduced fertility in
294 the F_1 generation and led to significantly less F_2 larvae compared to the naïve control (THD: z= -2.381,
295 p<0.05; Figure 3b). Therefore, paternal septic wounding but not paternal bacterial priming reduces the
296 fitness of the F_1 generation.

297 3.2 Transgenerational effects of priming in larvae

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298 ### 3.2.1 Larval priming does not affect fertility

299 Neither oral nor injection priming of larvae with spore supernatant or heat-killed bacteria,
300 respectively, significantly affected fertility compared to the control groups or the naïve individuals
301 (GLM: df=4, X^2=2.11, p=0.71, Figure S1).

302 ### 3.2.2 Oral priming affects development differently in treated and offspring generation

303 We monitored larval development after oral priming to discover potential additional costs and
304 benefits of this treatment besides changes in survival rate upon infection. In the treated parental
305 generation, there were significant differences in the pupation rates 21 dpo to 25 dpo (
306 **Figure 4**a). Larvae treated with *Bt407* supernatant, a bacterial strain that has been shown to not cause
307 any immune priming (Milutinović et al. 2014) and therefore served as a priming control, reached
308 pupation faster than the *Btt* primed group (z=-2.906, p=0.0102). There also was a trend towards
309 earlier pupation of the *Bt407* treated larvae compared to the naïve control (z=-2.28, p=0.059), while
310 the *Btt* primed group and naïve control did not differ (z=-0.875, p=0.65). Additionally, there were
311 differences in time until adult eclosion (GLMM: df=2, X^2= 17.52, p<0.001;

312 **Figure 4**b). At 28 dpo significantly more pupae from the *Bt407* priming control had eclosed than
313 from the *Btt* primed group (z= 2.98, p=0.008) and the naïve control (z= 3.802, p<0.001). Again, there
314 was no difference between the *Btt* primed and naïve control (z= 0.569, p=0.84).

315 We also observed the development in the F_1 generation to see if this was influenced by the parental
316 oral priming. Larvae, whose parents were exposed to spore culture supernatant from *Btt* or *Bt407*
317 developed significantly slower than offspring of the naïve control (GLMM: df=2, X^2= 16.14,
318 p<0.001; *Bt407*: z=3.83, p=0.002; *Btt*: z=3.832, p<0.001, Figure 5a). We found a similar effect for
319 the development until adult eclosion, which on average was reached earliest by the naïve group
320 (GLMM: df=2, X^2= 14.17, p<0.001; *Bt407*: z=-3.213, p=0.004; *Btt*: z=-3.199, p=0.004; Figure 5b)

321 ### 3.2.3 No survival benefits of oral TGIP for offspring generation

322 To test whether the exposure to spore supernatants led to a trans-generational priming effect, *i.e.*
323 increased offspring survival upon infection, larvae of the primed P_0 and the F_1 generation were orally
324 exposed to spores. In the primed P_0 generation, the challenge with *Btt* spores killed the larvae at a
325 significantly higher rate than the exposure to spores of *Bt407*, which served as the treatment control
326 (df=1, X^2=12.76, p<0.001; Figure S2). This, however was regardless of priming treatment, which did
327 not lead to any significant differences (df=2, X^2=0.63, p=0.73; Figure S2). This might be attributed to
328 overall relatively low mortality rate after challenge with only 10.8 % of all exposed larvae dying.
329 This probably was caused by the rearing of larvae in lose flour instead of flour discs for the period
330 between priming and challenge, because of which many larvae might have already had reached a pre-
331 pupal stage and stopped feeding.

332 Although mortality was higher, results for the offspring generation were similar (Figure S3). Again,
333 the bacterial challenge proved to cause significant mortality at high (df=1, X^2=96.63, p<0.001) and
334 low concentration of spores (df=1, X^2=47.1, p<0.001). Furthermore, survival depended on *Btt* spore
335 concentration as the higher dose led to significantly higher mortality (df=1, X^2=10.85, p<0.001). But,
336 no effect of parental priming was observed (df=2, X^2=0.69, p=0.71; Figure S3).

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337 **3.2.4 Transgenerational effects of injection priming in larvae**

338 In this part of the experiment we investigated potential effects of priming of larvae by injection on
339 their development and the development of their offspring. Nine days after the priming, significantly
340 less individuals from the control injection treatment had pupated compared to the naïve control
341 (X^2=8.466, p=0.003, Figure 6a). The addition of heat-killed bacteria to the injection reduced this
342 effect, resulting in only a trend towards later pupation in the *Bt* priming treatment compared to the
343 naïve control (X^2=3.74, p=0.053, Figure 6a). There was no significant difference in the pupation rate
344 between the *Bt* primed individuals and the injection control (X^2=1, p=0.317, Figure 6a). In the F_1
345 offspring generation we did not observe any effect of parental priming on the developmental speed,
346 as the eclosion rate was similar for all treatment groups at 27 dpo (GLMM: df=2, X^2=4.62, p=0.1).

347 We challenged the parental and offspring generation by injecting a potentially lethal dose of *Bt* at 19
348 dpo, i.e. five days after the priming procedures for the parental generation. As the majority of
349 mortality occurred within 24 hours of the bacterial injection, we did not use survival curves in the
350 analysis, but instead used the survival rate differences after seven days for our analysis.

351 In the P_0 generation priming did not lead to differential survival after the injection challenge, which
352 caused between 23 % and 27 % mortality (df=2, X^2=0.291, p=0.86). Finally, in the F_1 offspring
353 generation, the bacterial injection challenge caused significantly higher mortality than the injection
354 control (GLMM: df=1, X^2=244, p<0.001, Figure S4). However, also in this case parental priming did
355 not significantly impact survival as there were no significant differences in mortality rates between
356 the parentally primed group and the two controls (GLMM: df=2, X^2=0.037, p=0.98, Figure S4).

357 **4 Discussion**

358 *T. castaneum* is one of the rare species for which not only maternal but also paternal TGIP has been
359 observed (Roth et al. 2010; Roth et al. 2018). It is therefore important to further study this
360 phenomenon. One of the major open questions regarding paternal TGIP is, whether it is effective in
361 more than one subsequent generation and can be considered to be multigenerational. Additionally, it
362 is important to understand what the costs of TGIP are and if these are also transferred to later
363 generations. We therefore carried out bacterial priming and challenge experiments across three
364 generations using adult beetles.

365 We found that offspring of primed grandfathers survived a bacterial challenge significantly better
366 than offspring of grandfathers, which had received a priming control injection. Thus, paternal TGIP
367 is persistent for multiple generations at least until the F_2 generation. Astonishingly, the survival
368 advantage of the F_2 generation was at a similar level as observed in previous experiments for the
369 direct offspring (Eggert et al. 2014). We therefore did not see any dilution effect of this phenomenon
370 over subsequent generation. Furthermore, we witnessed indirect costs, not of TGIP itself, but of the
371 wounding procedure during the injection. These fitness costs became only visible after two
372 generations, when the offspring of fathers from the injection control group sired significantly less
373 offspring. In the present experiment, in contrast to previous studies (Roth et al. 2010; Eggert et al.
374 2014), were unable to detect a significant priming effect in the adult F_1 offspring after paternal
375 priming. This was likely due to an unusually high mortality in the injection control, maybe caused by
376 a bacterial contamination in the injection buffer that was used for all treatments, thereby reducing a
377 potential effect of priming.

378 Few studies have investigated the effects of TGIP beyond the first offspring generation. It has been
379 shown that viral silencing agents derived from an RNAi response can be inherited non-genetically

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380 from either parent and passed on for several generations (Rechavi et al. 2011). In parthenogenetic
381 *Artemia*, maternal exposure to bacteria provided the offspring with a survival benefit of bacterial
382 infection for all three tested offspring generations (Norouzitallab et al. 2015). Multigenerational
383 effects of paternal TGIP have been described in the pipefish, where due to male pregnancy contact
384 between father and offspring is much more pronounced than in our system (Beemelmanns and Roth
385 2017). Although, we are as of today unaware of the mechanisms behind paternal TGIP against
386 bacteria, we can assume that its multigenerational nature will strongly impact the evolution of
387 resistance and tolerance, depending on the costs, benefits and specificity of TGIP and the prevalence
388 of and therefore chances of repeated exposure to a parasite.

389 In the second part of this study, we investigated the transgenerational impact of immune priming via
390 two different infection routes in larvae, for which within life stage immune priming has been
391 previously demonstrated (Roth et al. 2009; Milutinović et al. 2014). Additionally to the survival after
392 bacterial challenge, we monitored fitness costs of larval priming, becoming apparent as either directly
393 reduced fertility or by slowing down developmental speed of the treated individual or its offspring.
394 As any form of immunity, also immune priming comes at a cost for the organism (Sadd and Schmid-
395 Hempel 2009; Schmid-Hempel 2005; Freitak et al. 2009). While in mosquitos a trade-off between
396 immune priming and egg production has been observed (Contreras-Garduño et al. 2014), we did not
397 find any effects of priming on fertility. Similar numbers of live offspring were produced across all
398 treatments for both priming methods. But we estimated fertility only from a short 48h reproduction
399 period and do not know how the immune priming might affect lifetime reproductive success. Also,
400 we provided the beetle with *ad libitum* food throughout the experiment, whereas limiting resources
401 can be necessary for uncovering trade-offs with immunity (Kutzer and Armitage 2016; Moret and
402 Schmid-Hempel 2000).

403 However, the oral priming of larvae led to differential speed in their development. Larvae, which had
404 received the priming control diet containing the supernatant from the *Bt407* culture reached pupation
405 considerably faster and emerged as adults earlier. In contrast, the treatment with *Btt* did not lead to
406 differential developmental time compared to the naïve larvae. The same effect was observed
407 previously by Milutinović et al. (2014). It is possible that the supernatant from the *Bt407* control
408 culture contained some nutrients that were transferred to the priming diet and helped the larvae to
409 speed up their development. The supernatant from the treatment *Btt* culture might not contain these
410 nutrients, due to differences in the bacteria. Alternatively, the necessity to mount an immune and
411 priming response, brought on by the exposure to the priming diet might mitigate the potential effect
412 of the additional nutrients.

413 In the offspring generation, development was strongly affected by parental larval treatment. Both,
414 offspring from the *Btt* primed group and the *Bt407* priming control took longer to pupate and also
415 emerge as adults. This is interesting because although *Bt407* does neither provide an immune priming
416 (Milutinović et al. 2014) nor is able to kill larvae upon ingestion (Milutinović et al. 2013), larvae
417 feeding on its spore supernatant still suffer these fitness costs. These results are in concordance with
418 observations in the mealworm beetle, where maternal priming prolonged larval development, while
419 paternal priming led to a reduction in larval body mass (Zanchi et al. 2011). For the injection
420 priming, we only observed within generation effects on the development. Here the wounding by the
421 injection was sufficient to cause the effect, because larval development was slowed down in the
422 injection of heat-killed bacteria as well as in the injection control treatment compared to the naïve
423 group. In the offspring generation, time until adult emergence was not affected by parental priming.
424 So far, we have no data regarding the development until pupation in this case.

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425 Increased developmental time during the larval and pupal stage can be considered a fitness cost.
426 Longer time spend during the larval stage is costly as it increases several risks. During the larval
427 stage the risk of infection is higher as only larvae can be infected orally with certain bacteria,
428 including *Btt*. Also, there is a higher risk of cannibalism, which happens regularly among larva
429 (Ichikawa and Kurauchi 2009) and at high densities smaller larvae might be less able to secure
430 sufficient food (Koella and Boëte 2002). Therefore, prolonged development should decrease
431 probability of survival and delay the start of reproduction. In this experiment we were unable to
432 confirm within-generation immune priming for either of the two used infection methods. This can
433 likely be attributed to the low overall mortality rates following the challenge, which is a problem
434 occasionally encountered in such experiments (see also Tate et al. 2017). However, both within-
435 generation priming methods have been shown to work consistently in our lab (Milutinović et al.
436 2014; Ferro et al. 2017; Futo et al. 2017).

437 We did not find any evidence of larval TGIP with the oral nor the injection protocol. For larval
438 priming by septic wounding with a pricking needle, it was observed that TGIP in larvae only
439 occurred in populations, which do not demonstrate within life stage immune priming (Khan et al.
440 2016), implying that they are incapable of developing and maintaining both forms of immune
441 protection. As beetles from our population have repeatedly been shown to possess larval within life
442 stage priming ability, this is a possible explanation for the absence of larval TGIP.

443 In conclusion, we observed that different ways of immune priming can have different effects on the
444 next generation, depending on the life stage and route of priming. These effects might not always be
445 beneficial, as parental treatment appeared to impact on offspring development, demonstrating
446 potential costs of immune priming that are paid by the next generation. These would remain
447 undetected if only the treated generation is studied. We therefore do not only need more studies on
448 mechanisms behind the different routes of immune priming, but also more experimental research
449 focusing on the evolutionary consequences of immune priming. This will help to clarify under which
450 circumstances this ability is favored over the evolution of resistance or tolerance (Tidbury et al. 2012;
451 Tate 2017). Such knowledge will be needed to advance methods of pest control, which strongly
452 depend on the use of bacterial products, e.g. toxins from *B. thuringiensis*.

453 **5 Conflict of Interest**

454 The authors declare that the research was conducted in the absence of any commercial or financial
455 relationships that could be construed as a potential conflict of interest.

456 **6 Author Contributions**

457 All authors contributed to the conception and design of the study; NS, MPS, KF and NK performed
458 the experimental work; NS performed the statistical analysis and drafted the manuscript. All authors
459 contributed to manuscript revision and read and approved the submitted version.

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638

639 **10 Data Availability Statement**

640 The datasets generated for this study can be found in the supplementary material.

641

642 **11 Figures**

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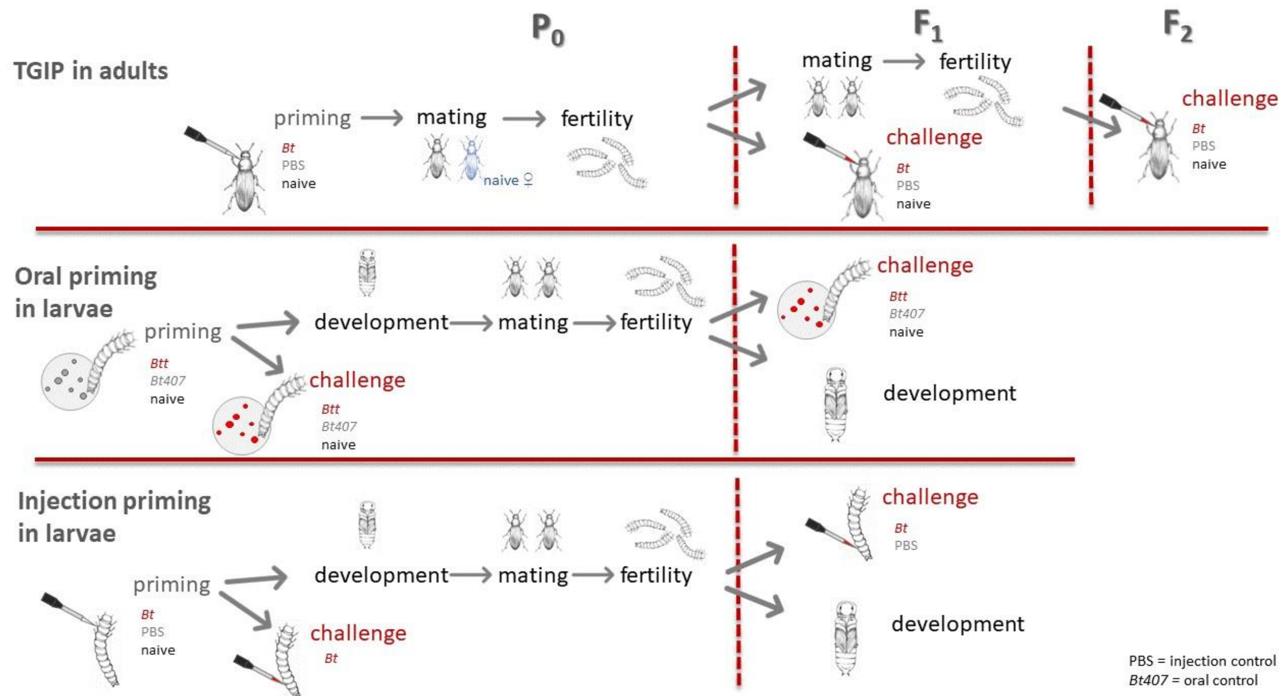
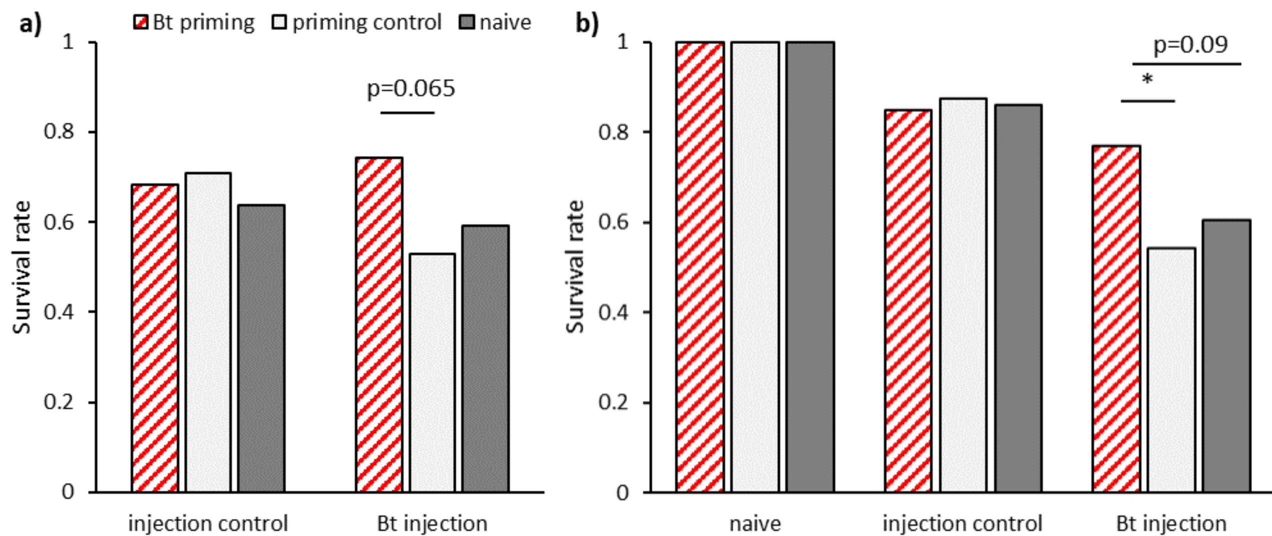


643

644 **Figure 1 Overview of the experimental design**

645



646

647 **Figure 2: Survival of bacterial challenge after paternal TGIP. a) Adult F1 generation after**
648 **parental priming (n=31-44) and b) adult F2 generation after grandparental priming (two**
649 **experimental blocks: n=16-42 and n=24-45). Asterisks indicate significant differences at p <**
650 **0.05.**

651

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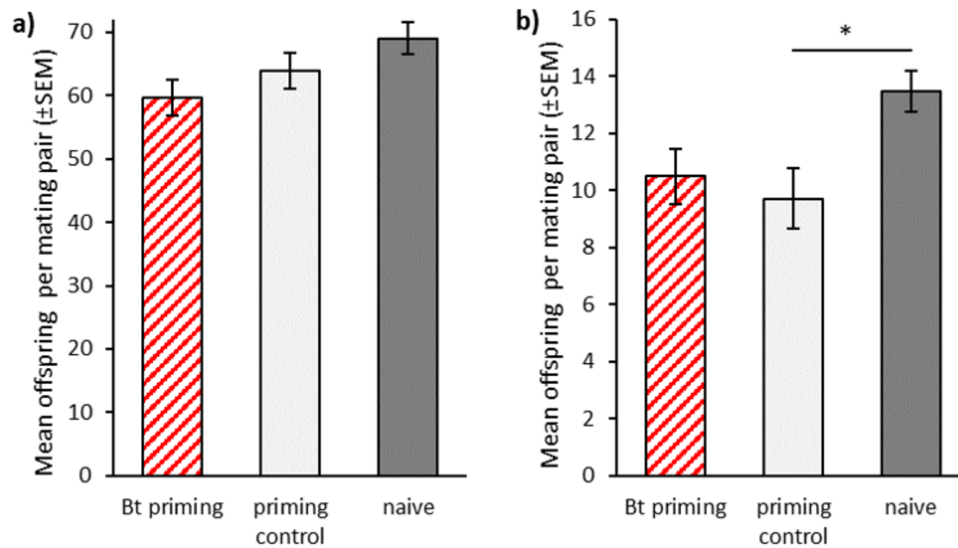
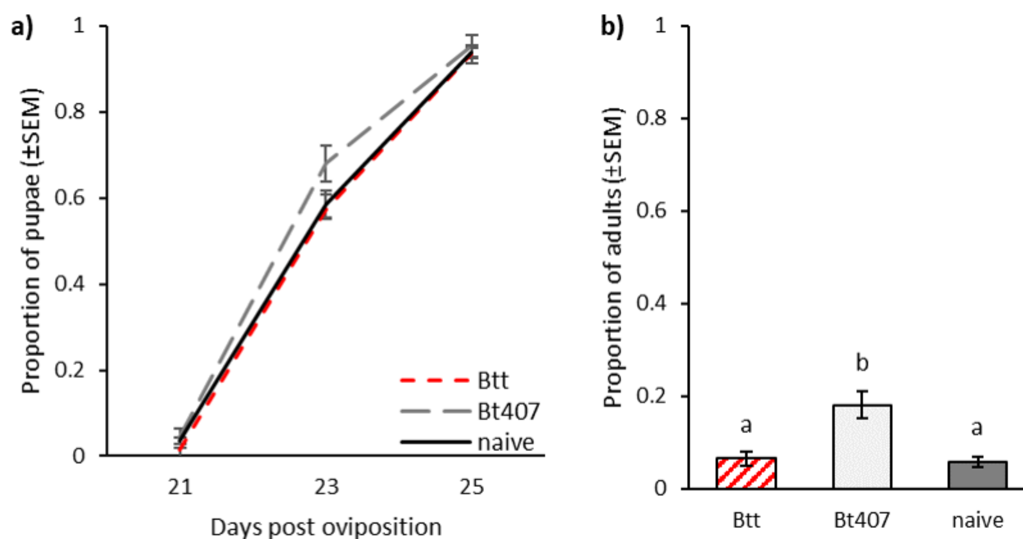


652

653 **Figure 3: Male fertility after injection priming of adults. a) Mean offspring produced by**
654 **primed males within 6 days (n=39-57) and b) mean offspring produced by offspring of primed**
655 **males within 48 h (n=29-53). Asterisk indicates significant differences at p < 0.05**

656



657
658 **Figure 4 Development after oral priming during larval stage (n=196-280). a) Pupation rate for**
659 **nine replicates b) Proportion of eclosed adults 28 dpo for nine replicates. Different letters**
660 **indicate significant differences at p<0.05**

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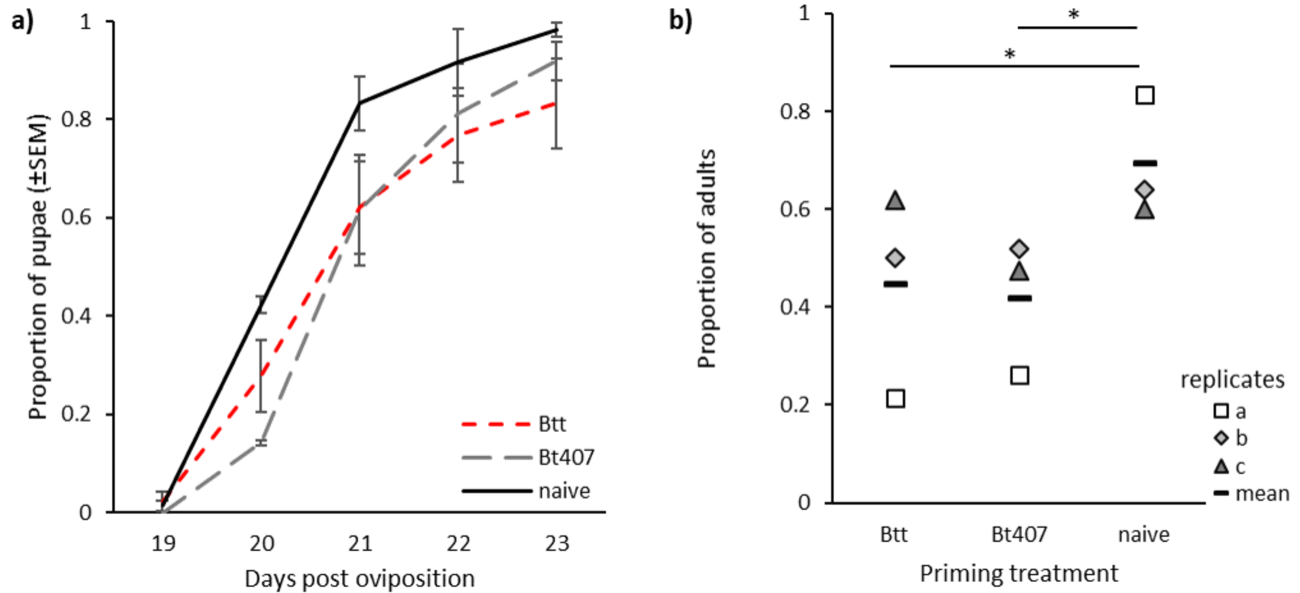
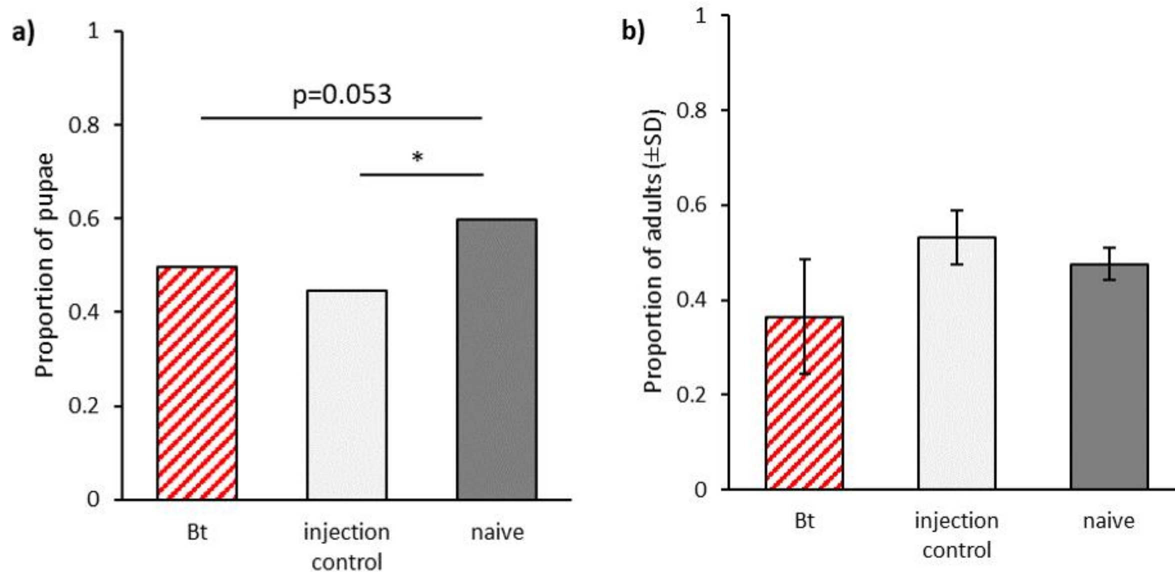
661



662

663 **Figure 5: Offspring development after parental oral priming during the larval stage (n=70-75).**
664 **a) Pupation rate b) Proportion of eclosed adults 28 dpo for three replicates. Asterisks indicate**
665 **significant differences at p<0.05**

666



667

668 **Figure 6: Development after parental injection priming during the larval stage. a) Proportion**
669 **of pupae for P_0 23 dpo (n=196). b) Proportion of eclosed adults for F_1 27 dpo for two**
670 **experimental blocks (n=72-103). Asterisks indicate significant differences at $p < 0.05$.**