PNAS
www.pnas.org

Supplementary Information for

Experimental Evolution of immunological specificity

Kevin Ferro, Robert Peuß, Wentao Yang, Philip Rosenstiel, Hinrich Schulenburg & Joachim Kurtz

Joachim Kurtz
Email: joachim.kurtz@uni-muenster.de

This PDF file includes:

Supplementary material and methods
Figs. S1 to S3
Tables S1 to S3
References for SI reference citations

Other supplementary materials for this manuscript include the following:

Datasets S1 to S7

www.pnas.org/cgi/doi/10.1073/pnas.1904828116

Supplementary Material and Methods.

Insects

T. castaneum (Cro1 population) were wild-collected in Slavonski Brod, Croatia in 2010 and allowed to adapt to laboratory conditions for at least 12 generations (approx. 12 months) before the start of the experiments. Beetles were raised on white flour (type 550) with 5 % brewer's yeast at 30 ºC, 70 % humidity, in a 12 h light/dark cycle.

Bacteria

The bacteria used for the infection experiments were *B. thuringiensis morrisoni var. tenebrionis* (*Btt*; BGSCID 4AA1 aquired from the Bacillus genetic stock center (BGSC)), *B. thuringiensis* (*Bt1*; DSM no. 2046), *B. thuringiensis yunnanensis* (*Bt2*; DSM no. 6073), *B. thuringiensis 407* (*Bt407*; kindly provided by Dr. Christina Nielsen-Leroux, Institut National de Recherche Agronomique, France), *Lactococcus lactis* (*Ll*; DSM no. 20481) and *Pseudomonas fluorescens* (*Pf*; DSM no. 50090) (Table S1 for details). For experiments, 50 µl of glycerol stocks containing lag-phase bacteria were added to 50 mL of LB medium in a 500 mL baffled Erlenmeyer flask and incubated at 30 ºC, 200 rpm overnight for 15 hours. The overnight cultures were pelleted at 4 ºC, 4.500 rpm for 15 minutes, the supernatant discarded, and the bacteria washed once in 20 mL phosphate buffered saline (PBS, Calbiochem®). The bacteria were resuspended in PBS and concentration was estimated with a Thoma counting chamber.

Selection protocol

We used approximately 10,000 2-3-week-old adult beetles as ancestral parental generation to produce animals for three different selection treatments: Specific Immunity (*'specific'*), Unspecific Immunity (‘*unspecific'*), Genetic Specificity (*'genetic'*) and two control treatments: Pricking Control (‘*pricking'*) and Untreated Control (‘*untreated'*). Every selection treatment was replicated six times while the controls were replicated three times (three selection treatments x six replicates + (two control treatments x three replicates) = 24 lines). In all selection treatments and controls each replicate contained 96 animals that were used for priming and challenge resulting in a total number of animals of 32,256 (24 lines x 96 animals x 14 Generations). Animals were randomly chosen out of pool of offspring from the former generation of that replicate and line, respectively. The specific selection treatment was used to select for the ability to raise a specific immune response upon homologous priming. Therefore, this line was primed and challenged with the same bacteria within generations, but different bacteria species across generations, to avoid transgenerational effects as described in Roth et al, (2010)(1) (Figure 1). Since we aimed with this selection on the trait of immune specificity, all six lines were independent of each other in terms of bacteria treatment throughout the selection process (Table S2). The unspecific selection treatment was used to select for unspecific immunity in the sense of a broad-range innate immune response (Figure 1). To achieve this, we primed and challenge with different bacteria within and across generations. As in the specific treatment, we never primed or challenged with a bacteria species that was used in the previous generation to avoid direct transgenerational priming effects

(as shown for T. castaneum in Roth et al., 2010). We included the genetic selection treatment to test for effects of resistance evolution against the bacteria used in the selection procedure and to serve as a control for our directional selection protocol. Hence, for each of the six bacteria species used in the experiment, one line was chosen for homologous priming and challenge within and across generations (e.g. replicate line GS6 was always primed and challenged with *Bt1*). To control for any effects towards repeated wounding, the pricking treatment was aseptically primed and challenged using sterile PBS (Calbiochem®). Finally, the untreated treatment was reared at densities like the wounded and infected selection treatments to control for effects of population size on the response to selection.

During the selection process we monitored survival, developmental speed and sex-ratio. Survival was always monitored twice; at first on the day of challenge to control for any larvae that died after priming and second, eight days after challenge to monitor the survival rate. Developmental speed in form of life stage (larvae, pupae or adult) and sex ratio was also monitored eight days after challenge (Figure S3 for a summary of the experimental plan).

At the beginning of the selection process, a randomized order was established for all lines from every treatment and repeated for every generation and phenotypic screen (Table S2). Only one line was started per day so that priming and challenge, respectively, was done on one day to ensure feasibility. Every generation started with the survivors of the former, by placing adult beetles on flour with yeast (5 % w/w) for two days. Adults were removed from the flour with a 710 µm sieve and the eggs further cultivated under given standard conditions for another 9 days. Then larvae were sieved with a 560 µm sieve, 96 larvae randomly chosen and individualized into 96-well plates containing flour and 5% (w/w) yeast. Four days later these larvae were primed with heat-inactivated bacteria that were prepared as described in Roth et al. (2009)(2). Briefly, bacteria were grown from a glycerin stock overnight for 15 h in 50 mL standard LB-Media and centrifuged for 15 min, 5000xg at 4°C and washed with PBS twice. After the last wash, the pellet was resuspended in 2 mL PBS and counted in a Thoma counting chamber to adjust the cell concentration to 1 x 109 cells per mL-1. Bacteria were then heat inactivated for 30 min at 90°C. To ensure a complete inactivation, a sub-sample of the heat-inactivated bacteria was cultivated on LB-Agar plates at 30°C to ensure complete inactivation. For priming, larvae were pricked laterally between the 2nd and 3rd to last posterior segment with a sterile dissecting needle (~10 µm) that was dipped into the heat-killed bacteria solution.

After this priming treatment, larvae were again individualized into 96-well plates containing flour and 5% (w/w) yeast. After an additional time span of four days, larvae were infected with life bacteria, which were prepared as described in Roth et al. (2009)(2). Briefly, bacteria were grown as for the priming experiments and cell concentration was adjusted to a LD^{20} concentration (Table S1 for specific bacteria concentration). To ensure viability, a sub-sample of every bacterial sample used in the pricking infection experiment was cultivated on LB-Agar and colony-forming units were counted. For infection, the larva was pricked dorsally between the 1st and 2nd segment into the dorsal vessel with a sterile dissecting needle (~10 µm) that was dipped into the live bacteria solution.

Screening for immune priming specificity of selection lines

After 7 and 14 generations of selection we performed a full reciprocal priming and challenge experiments with all selection treatments and replicates, respectively. Here, we monitored survival, developmental speed and the short-term fecundity of the surviving adults. We performed the priming and challenge with the following bacteria species: *Btt*, *Bt1* and *Pf*. We also included an untreated group in the design to control for priming and challenge effects on developmental speed and fecundity. To avoid transgenerational effects (1, 3) we used the unselected F2 offspring after generation 7 and 14, respectively, in this test for priming specificity. Briefly, a random selection of F1 adults (offspring of surviving animals of generation 7 or 14, respectively) were

incubated at standard conditions for two days. Larvae were cultivated as described previously. For priming, larvae were randomly assigned to one of the priming treatments. Every selection treatment and replicate were primed and challenge as in the selection process described above for generation 7. For every priming and challenge treatment we used 24 larvae in total. This resulted in a total of 384 larvae per selection treatment and replicate (24 larvae x 4 priming treatments x 4 challenge treatments = 384 larvae per line and replicate (x 24 lines) = 9,216 larvae in total).
For generation 14 we used an injection method to prime and challenge the larvae to expose them with controlled numbers of bacteria (described in (4)), resulting in the following changes to the previously described protocol. The injections were performed using the Nanoject IITM Auto-Nanoliter Injector equipped with two-step pulled, cut and back-filled glass capillaries. Every larva was injected with 18.4 nL of either a bacteria suspension or left untreated for priming and challenge, resulting in a dose of 18400 bacteria for all priming groups and a LD^{50}-inducing dose for all challenge groups Table S1 for specific bacteria concentrations and doses). For every priming and challenge treatment we used 18 larvae in total. This resulted in a total of 288 larvae per selection treatment and replicate (18 larvae x 4 priming treatments x 4 challenge treatments = 288 larvae (x 24 lines) = 6,912 animals). The order of the selection lines was kept as in the selection process for all three readout experiments. One month after eclosion of the surviving adults, three single mating pairs for all possible priming and challenge combinations were set up and left to produce offspring for a period of three days. Thereafter the adults were removed from the vials and the offspring larvae counted 17 days after start of the assay.

Statistical analysis of phenotypic data

All analyses of the phenotypic data were performed with R v3.4.1 (5) and RStudio v1.0.143 (6). Data for generation 7 and 14 was analysed separately. Censored survival 8 dpc was analysed with the "coxme" R package v2.2-10 (7), using a nested design to test for interaction effects of challenge, priming and selection treatment while treating the replicate lines as random factor. Censored survival data 8 dpc from the *genetic* selection treatment was analyzed with the "survival" R package v2.38 (8), using a nested design to test for interaction effects of challenge, priming and replicate line. The developmental speed was analysed by treating the developmental state 8 dpc as ordinal response variable (larvae<pupa<adult) to facilitate the usage of the "ordinal" R package v2015.6-28 (9). All analyses that tested for effects of bacteria species on developmental speed excluding the genetic selection treatment, starting from a full model, which contained challenge, priming and selection treatment as well as replicate line as random factor. For the analyses of the developmental data including the *genetic* selection treatments, no random factor was added to the model and the data for the different priming/challenge bacteria treatments were condensed into bacteria-treated and untreated control. The fecundity data was analyzed with the "lme4" R package v1.1-13 (10), fitting the data to a Gamma distribution. As the fecundity data for generation 14 was zero-inflated, it was analyzed using the pscl R package v1.5.1 (11) instead. In both cases the full model contained challenge, priming and selection treatment as well as replicate line as random factor. Stepwise simplification of the full model for all linear mixed models was performed to remove non-significant terms. The fitted models were tested for significant effects by Type 2 Wald X^2 tests included in the car R package v2.1-4 (12). Post-hoc contrasts of significant terms in the simplest model were performed using the lsmeans R package 2.26-3 (13) while correcting for multiple comparison via FDR ($p < 0.05$).

RNA extractions, library construction and Illumina sequencing

In order to identify the genetic basis for the responses to selection after 14 generations we performed a transcriptomic analysis with the same populations used for the phenotypic readout, excluding the genetic and pricking selection treatments. The 15-day old larvae were either primed

with heat-killed *Btt*, *Bt1*, *Pf* or left naive. RNA sampling was done as described in Behrens et al, (2014)(14). Briefly, for every replicate, 20 larvae were pooled six hours after priming (Figure S3). Pooled larvae were immediately snap frozen in liquid nitrogen and stored at -80ºC until further use. RNA was extracted using the mirVana miRNA Isolation Kit (Ambion) according to the protocol for total RNA extraction of the manufacturer, which has resulted in reliable extractions in previous studies (14, 15). The libraries for Illumina sequencing were prepared using the TruSeq RNA sample V2 Kit (Illumina) following the manufacturer's protocol. The libraries were then clustered on a cBot with the TruSeq PE Cluster Kit V4 (Illumina) with a final loading concentration of 10 pM according to the instructions of the manufacturer. The sequencing was performed with the TruSeq SBS Kit V4 on two lanes of the Illumina HiSeq 2500 yielding 2x125 bp paired reads per sample. The number of raw and filtered reads was similar for all sequenced populations (see Table S3 for details).

Transcriptomic analysis and correlation with phenotypic data

The quality of the raw reads was first checked with FastQC (16), after which the first five base pairs were removed using Trimmomatic 0.36 (17) to account for observed biases in sequence composition due to random hexamer priming (18). After this filtering step, STAR v2.5.3 (19) was used to map the reads to the Tribolium 5.2 reference genome downloaded from Beetlebase (20). The mapped reads were then converted from the .sam into the .bam format using SAM tools v0.1.19 (21) for downstream analyses. The .gff file used for mapping was then converted into a gtf reference file using the "gffread" function of cufflinks v2.2.1 (22). To obtain total gene counts from the mapped reads, the "htseq-count" function of HTSeq v0.7.2 (23) was used to count by gene ID, after which the individual assemblies were merged into one count table files by means of a custom-made R pipeline. Differentially expressed genes were identified with DeSeq2 v1.16.1 (24) under R and defined by a significance threshold of alpha = 0.05 and a False Discovery Rate of < 0.05. We analysed the RNASeq data according to two models: Model one compared the primed with the naive conditions for each selection treatment (testing for priming-induced changes), whereas model two compared the naive conditions for the specific and unspecific selection treatments to the untreated control treatment (testing for baseline differences in gene expression). Venn diagrams were generated by using the R package VennDiagram. Heatmaps were generated using the "pheatmap" R package. Only genes significantly differentially expressed compared to naive controls for at least one selection/priming treatment combination were considered. Genes were clustered using the Euclidean distance method, without clustering of the treatment combinations. The association of functional terms and GO term enrichment analyses were performed using the DAVID bioinformatics resource v6.8 and Version 5.2 of the T. castaneum genome annotation using the default settings with additional Benjamini-Hochberg correction and Fishers exact test for significance where appropriate (25, 26). Genes for which no annotation information could be found via DAVID were additionally screened with a Blastn (27) and Beetlebase search to facilitate interpretation of the results (20).
Lastly, the absolute expression values for the DEG identified in the DESeq2 analysis were correlated with the corresponding data for survival rates for all primed groups. For each gene, a Kendall test was performed to determine statistical significance at a threshold of $p = 0.05$. Because we correlated a set of gene expression data to one dataset of survival data, we corrected our analyses for multiple testing via the Benjamini-Hochberg method at a threshold of $p = 0.05$.

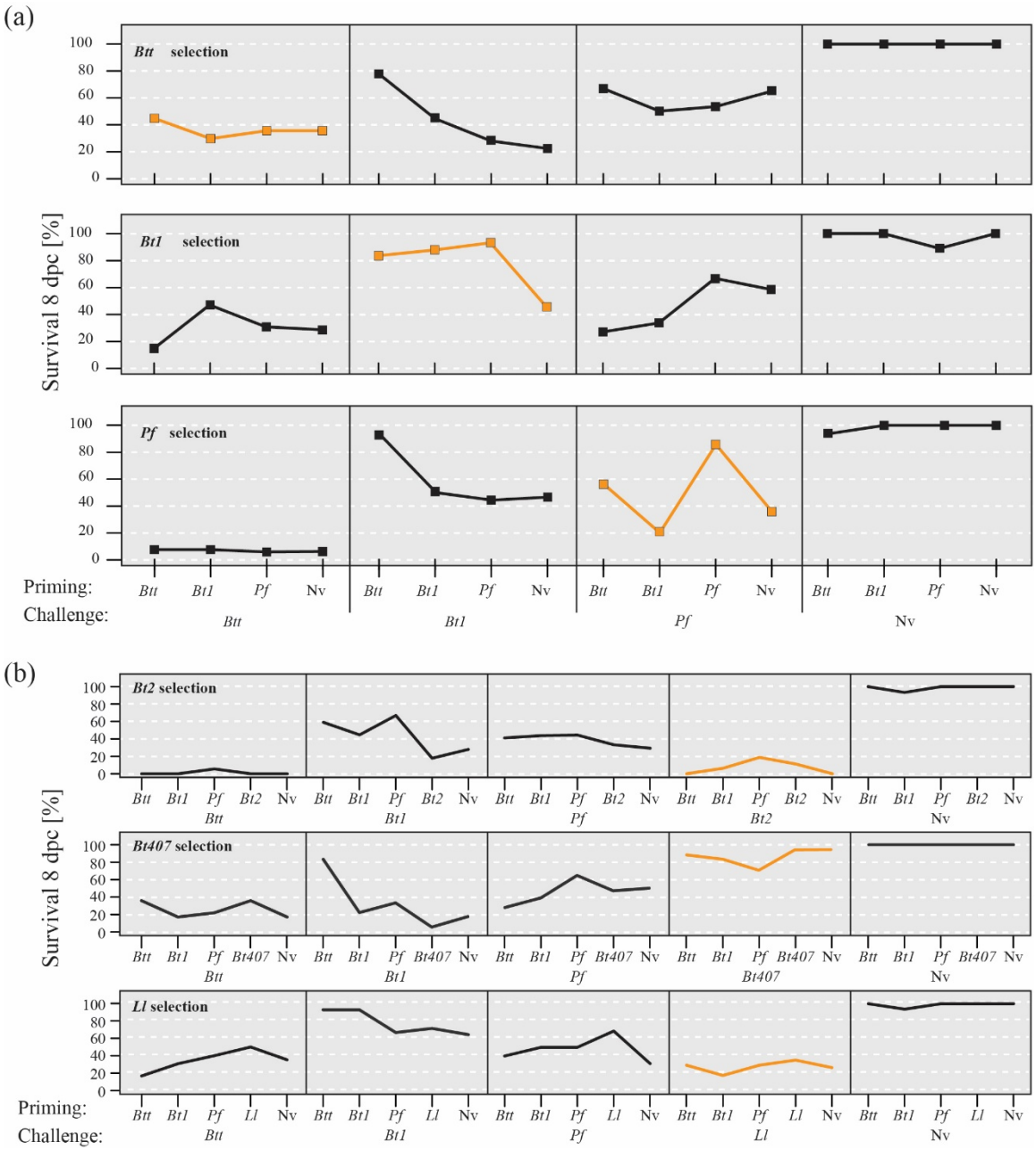

Fig. S1: Survival rates for genetic selection treatment by challenge bacteria and corresponding replicate line. The matching challenge bacteria/bacteria used for resistance evolution combination is highlighted in orange. The matching priming bacteria/bacteria used for resistance evolution is indicated for each line. (A) bacteria used in post-selection specificity screen (B) bacteria species only used during experimental evolution. n = 18 for all replicate lines.

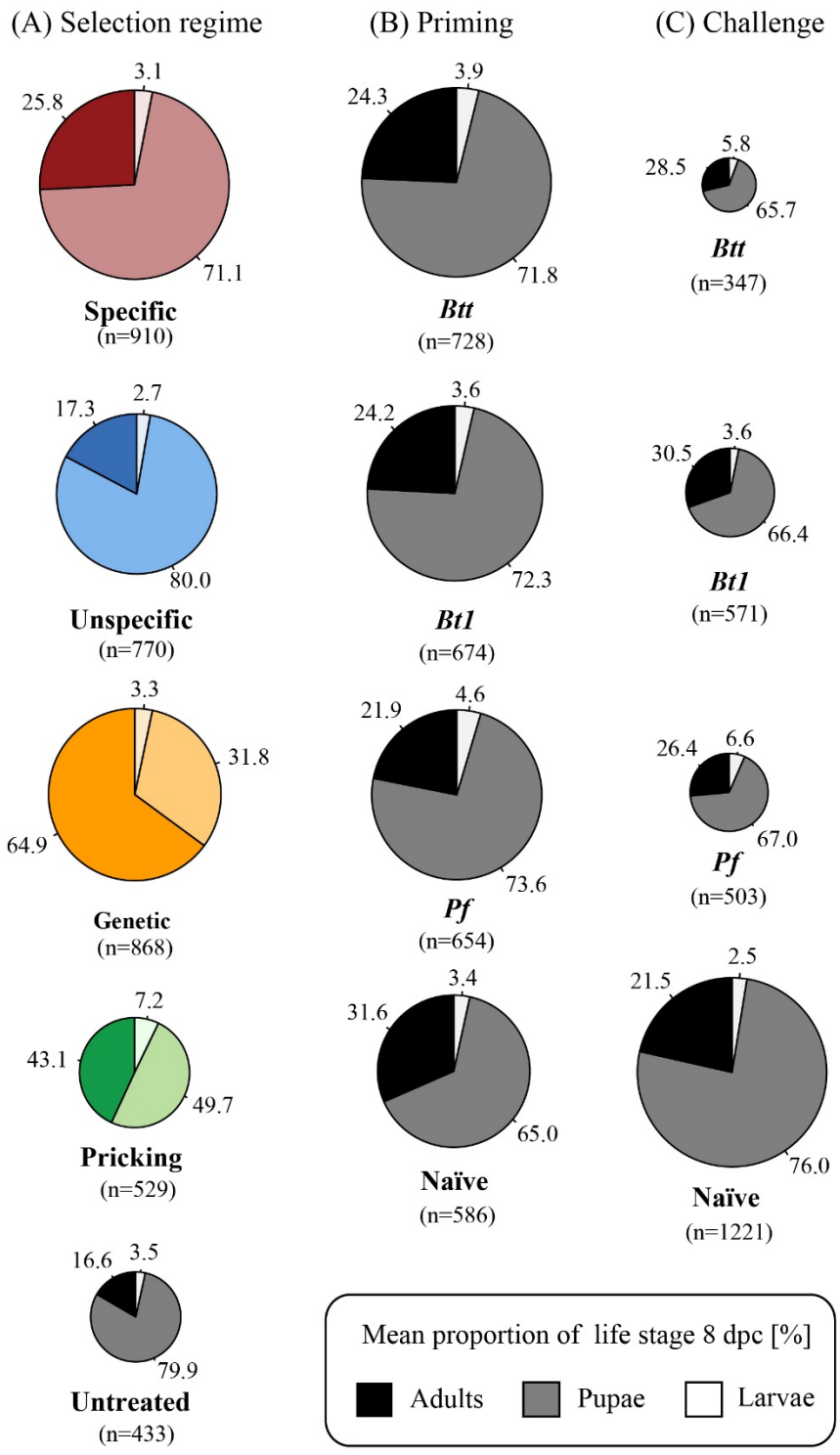

Fig. S2: Developmental rates of all selection treatments 8 days after priming and challenge. Proportion of larvae, pupae and adults by (A) selection treatment, (B) priming treatment and (C) challenge treatment. Size of pie chart corresponds to the total sample size per treatment. Lightest colour hue = larvae, medium colour hue = pupae, darkest colour hue = adults.

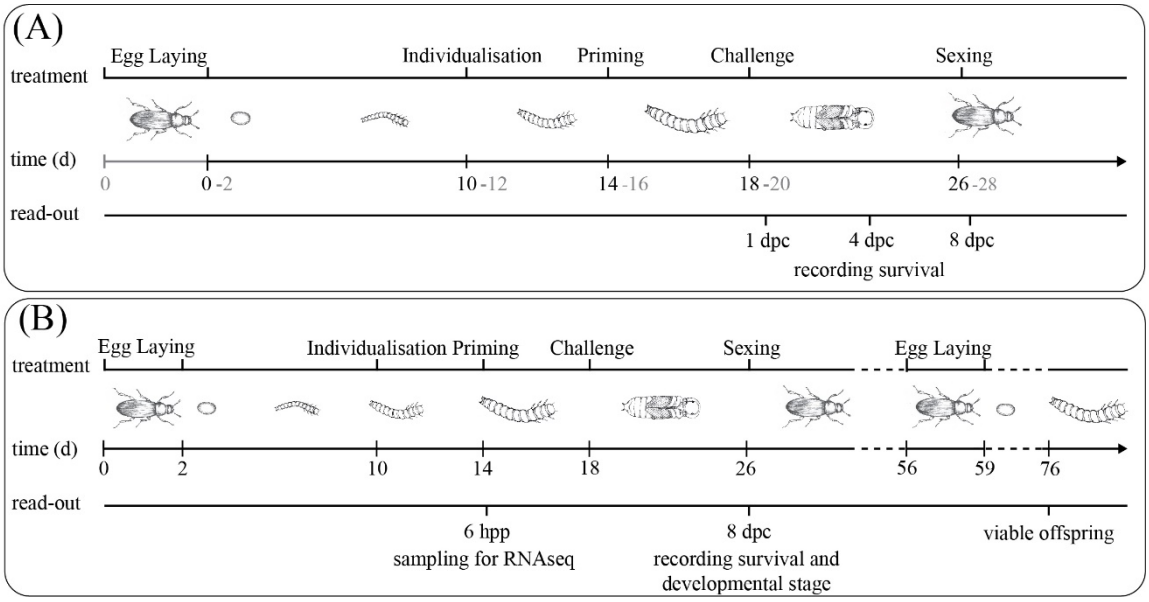

Fig. S3: Additional experimental design and timeline of selection protocol. (A) Overview of the selection protocol starting with the adults of the previous generation. (B) Overview of the phenotypic screening protocol starting with adults of an unselected F1 to avoid transgenerational effects. hpp = hours past priming, dpc = days past challenge. Drawings of *T. castaneum* by Sina Pflügge.

Table S1: Bacteria species used in selection treatments with the respective LD20 for pricking and LD50 for injections.

Bacteria species	$LD_{(20)}$ pricking (in cells/mL)	$LD_{(50)}$ injection (in cells/ml and abs. dose)	Source	ID
Bacillus thuringiensis morrisoni var. tenebrionis (*Btt*)	$1x10^{10}$	$5x10^{7}$ or 920 cfu	*Bacillus* genetic stock center (BGSC, USA)	4AA1
Bacillus thuringiensis (*Bt1*)	$1x10^{9}$	$1x10^{7}$ or 184 cfu	Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, FRG)	2046
Bacillus thuringiensis yunnanensis (*Bt2*)	$5x10^{9}$	$5x10^{7}$ or 920 cfu	Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, FRG)	6073
Bacillus thuringiensis 407 (*Bt407*)	$1x10^{9}$	$1x10^{7}$ or 184 cfu	Dr. Christina Nielsen-Leroux, Institut National de Recherche Agronomique, France	Bt407
Pseudomonas fluorescens (*Pf*)	$1x10^{7}$	$5x10^{5}$ or 9 cfu	Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, FRG)	50090
Lactococcus lactis (*Ll*)	$1x10^{7}$	$1x10^{10}$ or 184000 cfu	Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, FRG)	20481

Table S2: Selection treatments in their randomized order with corresponding bacteria for priming (P) and challenge (C) over 14 generations. Six replicates of selection treatments; "Specific Immunity" (S1-6), "Genetic Specificity" (G1-6) and "Unspecific Immunity" (U1-6) were primed and challenge with six different bacteria species: Bacillus thuringiensis morrisoni var. tenebrionis (1), Lactococcus lactis (2), Bacillus thuringiensis yunnanensis (3), Bacillus thuringiensis (4), Pseudomonas fluorescens (5), Bacillus thuringiensis 407 (6). Three replicates of control treatment; "Pricking control" (P1-3) and "Untreated Control" (C1-3) were only wounded (w) or left naïve (n), respectively.

	Generations													
Line	**1.**	**2.**	**3.**	**4.**	**5.**	**6.**	**7.**	**8.**	**9.**	**10.**	**11.**	**12.**	**13.**	**14.**
	P C	**P C**	**P C**	**P C**	**P C**	**P C**	**P C**	**P C**	**P C**	**P C**	**P C**	**P C**	**P C**	**P C**
U5	4 3	6 1	3 5	4 1	3 5	2 4	1 6	1 2	4 3	3 1	6 3	2 5	5 4	1 6
G3	1 1	1 1	1 1	1 1	1 1	1 1	1 1	1 1	1 1	1 1	1 1	1 1	1 1	1 1
U1	1 4	5 6	4 3	2 1	6 3	4 6	1 3	6 4	1 5	2 1	4 3	3 2	6 4	2 5
S5	3 3	6 6	2 2	1 1	3 3	5 5	4 4	5 5	3 3	2 2	1 1	4 4	5 5	2 2
C2	n n	n n	n n	n n	n n	n n	n n	n n	n n	n n	n n	n n	n n	n n
P1	w w	w w	w w	w w	w w	w w	w w	w w	w w	w w	w w	w w	w w	w w
C3	n n	n n	n n	n n	n n	n n	n n	n n	n n	n n	n n	n n	n n	n n
S2	5 5	2 2	4 4	3 3	4 4	5 5	1 1	2 2	6 6	4 4	5 5	3 3	1 1	4 4
S4	2 2	1 1	3 3	6 6	5 5	4 4	2 2	1 1	5 5	4 4	3 3	6 6	1 1	5 5
G2	5 5	5 5	5 5	5 5	5 5	5 5	5 5	5 5	5 5	5 5	5 5	5 5	5 5	5 5
S1	1 1	3 3	5 5	4 4	2 2	6 6	2 2	4 4	1 1	3 3	2 2	5 5	6 6	4 4
G4	3 3	3 3	3 3	3 3	3 3	3 3	3 3	3 3	3 3	3 3	3 3	3 3	3 3	3 3
G6	4 4	4 4	4 4	4 4	4 4	4 4	4 4	4 4	4 4	4 4	4 4	4 4	4 4	4 4
G5	2 2	2 2	2 2	2 2	2 2	2 2	2 2	2 2	2 2	2 2	2 2	2 2	2 2	2 2
C1	n n	n n	n n	n n	n n	n n	n n	n n	n n	n n	n n	n n	n n	n n
P2	w w	w w	w w	w w	w w	w w	w w	w w	w w	w w	w w	w w	w w	w w
P3	w w	w w	w w	w w	w w	w w	w w	w w	w w	w w	w w	w w	w w	w w
U3	1 6	2 5	2 1	3 5	3 4	6 2	3 4	6 3	1 6	2 5	3 6	5 4	4 3	2 1
G1	6 6	6 6	6 6	6 6	6 6	6 6	6 6	6 6	6 6	6 6	6 6	6 6	6 6	6 6
S6	5 5	3 3	6 6	2 2	1 1	4 4	3 3	5 5	6 6	4 4	3 3	1 1	5 5	6 6
U4	5 3	4 2	6 3	5 4	2 1	3 6	3 5	6 2	5 3	1 6	2 4	4 6	5 2	3 1
U6	2 1	4 5	2 3	1 2	5 6	1 5	2 6	3 1	2 4	6 3	1 4	5 2	3 5	4 6
S3	5 5	3 3	1 1	5 5	6 6	3 3	2 2	5 5	4 4	1 1	5 5	2 2	4 4	5 5
U2	5 2	1 3	6 4	4 6	2 1	2 4	1 5	2 3	5 2	3 1	4 6	6 1	1 4	3 5

Table S3: Number of raw and filtered reads for each RNASeq sample.

Sample_ID	Selection treatment	Priming treatment	Replicate	Raw reads	Past filter reads
F11257	Specific	Naive	1	17101870	14475578
F11258	Untreated	Naive	2	21569966	18619779
F11259	Unspecific	Pf	5	18478935	15759651
F11260	Specific	Btt	3	21192250	18186932
F11261	Specific	Naive	2	16574639	14237332
F11262	Untreated	Btt	2	18035600	15513136
F11263	Unspecific	Naive	3	18354895	15622373
F11264	Unspecific	Naive	5	19820302	16758696
F11265	Untreated	Bt1	1	20023693	17124754
F11266	Specific	Naive	4	18902423	16004592
F11267	Unspecific	Bt1	4	19175742	16492101
F11268	Untreated	Pf	3	20076880	17067642
F11269	Specific	Bt1	3	18742016	15955138
F11270	Unspecific	Bt1	5	18156093	15477315
F11271	Unspecific	Pf	4	17506157	14793190
F11272	Untreated	Pf	2	18450521	16051034
F11273	Unspecific	Naive	1	17489506	15350948
F11274	Unspecific	Bt1	2	21199546	18565423
F11275	Untreated	Btt	1	18712090	16070742
F11276	Specific	Bt1	2	16177083	13837537
F11277	Unspecific	Bt1	3	17663797	15207365
F11278	Specific	Pf	2	16919118	14712776
F11279	Unspecific	Pf	6	16112388	13916926
F11280	Unspecific	Btt	4	18775004	16245844
F11281	Unspecific	Btt	3	19510555	16982117
F11282	Untreated	Naive	3	20529067	18172723
F11283	Unspecific	Naive	6	17319600	15011569
F11284	Unspecific	Pf	3	18435496	16219143
F11285	Specific	Btt	2	22149669	19162768
F11286	Specific	Btt	5	17338849	15263513
F11287	Specific	Pf	5	20556815	18192522
F11288	Specific	Pf	6	20622154	18051480
F11289	Untreated	Bt1	3	21861007	19251720
F11290	Untreated	Pf	1	20118649	17641883
F11291	Unspecific	Naive	2	20825873	18461582
F11292	Specific	Btt	1	21844596	19208800
F11293	Specific	Btt	6	22146821	19477418
F11294	Specific	Naive	3	18791333	16492940
F11295	Unspecific	Btt	6	17693155	15508047
F11296	Specific	Pf	1	19144970	16759719
F11297	Specific	Bt1	5	19273471	16984925
F11298	Untreated	Btt	3	19452143	17205364
F11299	Specific	Btt	4	20796368	18293350
F11300	Specific	Bt1	4	23756265	20815720
F11301	Specific	Pf	4	19541200	17022709
F11302	Unspecific	Bt1	1	21321671	18427775
F11303	Specific	Bt1	1	15379196	13281893
F11304	Unspecific	Bt1	6	17973497	15527177
F11305	Specific	Pf	3	15464630	13377588
F11306	Untreated	Bt1	2	19735410	17346570
F11307	Unspecific	Btt	2	17996638	15572477
F11308	Unspecific	Naive	4	19137487	16785253
F11309	Specific	Bt1	6	16216910	14002141
F11310	Unspecific	Pf	1	23100848	20237566
F11311	Untreated	Naive	1	16958991	14757582
F11312	Specific	Naive	6	17976581	15505967
F11313	Unspecific	Btt	5	18526140	15991217
F11314	Specific	Naive	5	24968554	21620387
F11315	Unspecific	Pf	2	24649747	21573002
F11316	Unspecific	Btt	1	24594657	21331917
Average				**19348659**	**16792755**

Additional data table S1: Results of phenotypic screen of survival, developmental time and adult fecundity after priming and challenge for generation 7 (separate file).

Additional data table S2: Full report of statistical analyses of phenotypic data for generation 7 (separate file).

Additional data table S3: Full report of statistical analyses of phenotypic data for generation 14 (separate file).

Additional data table S4: Results of phenotypic screen of adult fecundity after priming and challenge for generation 14 (separate file).

Additional data table S5: List of DEG by pairwise comparison between priming and selection treatments (separate file).

Additional data table S6: List of annotated DEG for each section of Venn diagrams shown in Figure 3 (separate file).

Additional data table S7: List of GO terms for each section of Venn diagrams shown in Figure 3 (separate file).

References

1. Roth O, et al. (2010) Paternally derived immune priming for offspring in the red flour beetle, Tribolium castaneum. J Anim Ecol 79(2):403–413.
2. Roth O, Sadd BM, Schmid-Hempel P, Kurtz J (2009) Strain-specific priming of resistance in the red flour beetle, Tribolium castaneum. Proc R Soc B Biol Sci 276(1654):145–151.
3. Eggert H, Kurtz J, Diddens-de Buhr MF (2014) Different effects of paternal trans-generational immune priming on survival and immunity in step and genetic offspring. Proc R Soc B Biol Sci 281:20142089.
4. Ferro K, et al. (2017) Cu,Zn superoxide dismutase genes in Tribolium castaneum: Evolution, molecular characterisation, and gene expression during immune priming. Front Immunol 8(DEC). doi:10.3389/fimmu.2017.01811.
5. R Core Team (2017) R: A language and environment for statistical computing. Available at: https://www.r-project.org/.
6. R Studio team (2015) R-Studio: Integrated Development for R. Available at: https://www.rstudio.com/.
7. Therneau TM (2018) coxme: Mixed Effects Cox Models. R package version 2.2-10. Available at: https://cran.r-project.org/package=coxme.
8. Therneau TM (2015) A Package for Survival Analysis in S. Available at: https://cran.r-project.org/package=survival.
9. Christensen RHB (2018) Regression Models for Ordinal Data. R package version 2018.4-19. Available at: http://www.cran.r-project.org/package=ordinal/.
10. Meachler M, Bolker B, Walker S (2015) Fitting Linear Mixed-Effects Models Using lme4. J Stat Softw 67(1):1–48.
11. Zeileis A, Kleiber C, Jackman S (2008) Regression Models for Count Data in R. J Stat Softw.
12. Fox J, Weisberg S (2011) An {R} Companion to Applied Regression (Sage, Thousand Oaks, CA). Second Available at: http://socserv.socsci.mcmaster.ca/jfox/Books/Companion.
13. Lenth R V. (2016) Least-Squares Means: The R Package lsmeans. J Stat Softw 69(1):1–33.
14. Behrens S, et al. (2014) Infection routes matter in population-specific responses of the red flour beetle to the entomopathogen Bacillus thuringiensis. BMC Genomics 15(1):445.
15. Greenwood JM, et al. (2017) Oral immune priming with Bacillus thuringiensis induces a shift in the gene expression of Tribolium castaneum larvae. BMC Genomics 18(1):329.
16. Andrews S (2010) FastQC: a quality control tool for high throughput sequence data.
17. Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics 30(15):2114–2120.
18. Hansen KD, Brenner SE, Dudoit S (2010) Biases in Illumina transcriptome sequencing caused by random hexamer priming. Nucleic Acids Res 38(12):1–7.
19. Dobin A, et al. (2013) STAR: Ultrafast universal RNA-seq aligner. Bioinformatics 29(1):15–21.
20. Kim HS, et al. (2009) BeetleBase in 2010: Revisions to provide comprehensive genomic information for Tribolium castaneum. Nucleic Acids Res 38(SUPPL.1):437–442.
21. Li H, et al. (2009) The Sequence Alignment/Map format and SAMtools. Bioinformatics 25(16):2078–2079.
22. Trapnell C, et al. (2013) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc 7(3):562–578.
23. Anders S, Pyl PT, Huber W (2015) HTSeq-A Python framework to work with high-throughput sequencing data. Bioinformatics 31(2):166–169.

24. Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15(12):1–21.
25. Huang DW, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4(1):44–57.
26. Huang DW, Sherman BT, Lempicki RA (2009) Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 37(1):1–13.
27. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215(3):403–10.