Supplementary methods

Study insects and bacteria.

*Tribolium castaneum* (strain Cro1) were wild-collected in Croatia in 2010 (see 1) and adapted to laboratory conditions for at least 12 generations before the start of the experiments. Beetles were raised on organic wheat flour (Alnatura type 550) with 5 % brewer’s yeast at 30 °C, 70 % humidity, in a 12 hour light/dark cycle. *Drosophila melanogaster* (1_4WS) originated from inseminated females that were wild-collected in Münster, Germany in 2008 and allowed to adapt to laboratory conditions for at least 50 generations before the start of the experiments. These species have been estimated to have last shared a recent common ancestor around 300-327 million years ago (2, 3). Flies were kept on fly food (1.79 % brewer’s yeast; 3.57 % malt extract; 7.14% corn flour; 1 % soy flour; 0.4 % sugar beet syrup; 1.07 % agar; 0.14 % Nipagin; 0.4 % propionic acid) at 25°C, 70 % humidity, in a 12 h light/dark cycle, with non-overlapping generations.

The bacteria used for the infection experiments were *Bacillus thuringiensis morrisoni var. tenebrionis* (*B. thuringiensis*; BGSCID 4AA1) acquired from the Bacillus genetic stock center (BGSC), and *Escherichia coli* (DSM no. 498) and *Pseudomonas fluorescens* (DSM no. 50090) which were both acquired from the German collection of microorganisms and cell cultures (DSMZ). For experiments, 50 µl of bacteria glycerol stocks were added to 50 mL of LB medium in a 500 mL baffled Erlenmeyer flask and incubated at 30 °C, 200 rpm for 15 hours. Bacteria were washed in phosphate buffered saline (PBS, Calbiochem®) at 4 °C, 2,151 rcf, 15 min) and resuspended with 2 mL PBS and their concentration estimated with a Thoma counting chamber, and the concentration was adjusted as necessary.

Dscam1 expression in *D. melanogaster* and *T. castaneum*.

For both insect species, samples for gene expression profiling consisted of three replicate pools of whole body or tissue samples, each containing ten individuals.
Dscam1 expression in D. melanogaster.

For D. melanogaster we used whole body samples of eggs, first and third instar larvae, pupae, and adults were used. In addition we sampled fat body, haemocytes and brain from third instar larvae and fat body and brain from adults. We also sampled testes and ovaries from adults. To produce the samples, flies (4-5 days post pupal eclosion) were allowed to lay eggs for two hours on agar plates (1.5 % agar; 1 % vinegar) covered in a thin layer of yeast. The eggs were washed off the plates with PBS and three replicates of ten 12-14 hour old eggs were frozen. The flies were allowed to lay eggs for an additional 10 hours, after which time the eggs were washed off the plates with PBS and added to food vials at a constant density (methods after 4) and allowed to develop. Whole body samples of first instar larvae (35-45 hours after egg laying), third instar larvae (120-132 hours after egg laying), pupae (156-168 hours after egg laying), and adults (14 days after egg laying; i.e. around 4 day old adults) were used. The haemolymph from third instar larvae was obtained by carefully pulling animals apart on a glass slide without disrupting the gut. For fat body dissection third instar larvae were pulled apart by the mouth hooks and cuticle and the fat body was separated from other tissues. For adults, the fat body was obtained by removing the abdomen from the body; the gonads were discarded and the fat body tissue was collected into 50 µl of ice-chilled Drosophila Ringer’s solution (182 mM KCl; 46 mM NaCl; 3 mM CaCl2; 10 mM Tris•HCl; (5)) and shock frozen. Ovary and testis sampling were performed in the same manner as for the fat body but with an additional centrifugation step. All samples were immediately centrifuged after dissection (4 °C, 23 rcf, 5 min), the supernatant was discarded and the samples were snap frozen in liquid nitrogen and stored at -80 °C until RNA extraction. All samples from the pupal and adult stages, other than testes and ovaries, contained 5 males and 5 females.
For RNA extraction a tissue specific volume of TRIzol reagent (Ambion, U.S.A) was added to the frozen homogenised tissue samples, incubated for 10 min at room temperature (RT) and vortexed two times during incubation. To ensure complete lysis of the fat body, samples were first incubated with 250 µl TRIzol with an additional shock freezing and sonification step (5-10 min) before adding another 250 µl TRIzol and 10 min incubation at RT. Samples were centrifuged at 4 °C, 94 rcf for 10 min. The supernatant was transferred to a new tube and mixed with 100 µl of chloroform and incubated for 15 min at RT. Samples were centrifuged at 4 °C, 9,391 rcf for 15 min. The upper aqueous phase was used to isolate RNA with the SV Total RNA Isolation System (Promega) according to the manufacturer instructions, which included a DNase I digestion step. To optimise RNA concentration, the purified RNA samples were reduced to a volume of approximately 30 µl by vacuum-drying at 45 °C for 45 min. Samples were stored at -80 °C until use. For reverse transcription of RNA, SuperScript III™ (Invitrogen) was used according to the manufacturer’s instructions using random hexamer primers and a tissue specific amount of RNA as a template.

The resulting cDNA was used for qPCR analyses using gene-specific primers (Table S1). Where genes contained more than one exon, primers were designed such that the forward or reverse primer spanned an intron. Amplification efficiencies (E) of the primer pairs were determined with five dilutions (undiluted, 1:10, 1:100, 1:1,000, 1:10,000) of template cDNA, where \( E = 10^{\frac{1}{\text{slope}}} \). The qPCR was performed in a 384-well plate format, with a total reaction volume of 10 µl in each well. From each cDNA sample two technical replicate qPCR reactions were performed using the Kapa SYBR® Fast qPCR Mastermix according to the manufacturer’s instructions. The reaction was run on a LightCycler480 (Roche) using the following protocol: 95 °C for 5 min, followed by 40 cycles of annealing and amplification at 60 °C for one min and denaturation at 95°C for 15 sec. As a final step the products were heated up to 95 °C with continuous fluorescence measurements to obtain the melting curves.
and subsequently cooled to 40 °C. The crossing point (Cp) values (see raw data) were calculated using the Fit Point method using a Noise Band threshold of 10, with the LightCycler®480 software and the average Cp values from each of two technical replicates were used for analyses. \textit{Dscam1} expression for each sample was calculated relative to the geometric mean of two reference genes (ribosomal protein 49 (\textit{Rp49}) and ribosomal protein 13a (\textit{RpL13a})), used in the formula: \( E^{(reference)} - E^{(target)} = \Delta ct \). The genes \textit{RpL13a} and \textit{Rp49} were chosen as reference because they have been found to have relatively stable expression across treatments, life history stages and tissues (e.g., 6, 7-9); however it is important to recognise that it is difficult to find universally suitable reference genes (9). Although across tissues and life history stages, the Ct values of the two reference genes were tightly correlated with each another (\textit{D. melanogaster}: \( r^2 = 0.991, \text{df} = 34, p < 0.0001 \); \textit{T. castaneum}: \( r^2 = 0.986, \text{df} = 37, p < 0.0001 \)), it can be expected that reference genes do not necessarily show identical expression across different tissues and life history stages therefore we discuss the results in terms of expression of \textit{Dscam1} relative to the mean expression of the two reference genes.

\textit{Dscam1} expression in \textit{T. castaneum}.

A similar experimental set up was used for \textit{T. castaneum}: whole body samples of eggs, ten and fifteen day-old larvae, three day-old pupae and seven day-old adults were used. In addition we sampled fat body, haemocytes and brain from fifteen day-old larvae and from seven day old adults, and the testes and ovaries from adults. In detail, to produce the samples, two to three week old adult beetles were allowed to lay eggs for 24 hours. Three replicates of ten eggs were collected and frozen, and the remaining eggs were further cultivated as described above. After ten days, larvae were individualised in 96-well microtitre plates containing \textit{ad libitum} flour and 5 % yeast and incubated until sampling. Haemolymph was taken by pricking the larvae and adults with a fine needle (2 - 10 µm Ø) between the head and thorax. Clear haemolymph was collected with 1 µl glass capillaries (Hirschmann, Germany).
and immediately transferred into 50 µl ice-chilled Schneider’s insect medium (Sigma) containing 10 % fetal calf serum (FCS). For fat body dissections, larvae were cooled on ice and transferred to a microscope slide with a drop of PBS. Larvae were cut transversally at the first and last segment. The gut was pulled out and the fat body was then squeezed out with forceps and collected in 50 µl ice-chilled PBS. For adult fat body dissection, equal numbers of males and females were cooled and decapitated. The gut and gonads were removed and the fat body was collected in 50 µl ice-chilled PBS. Testes were sampled by cooling the beetle and cutting the abdomen by cutting beneath the last pair of legs on the ventral side. After transfer into a drop of PBS the testes were squeezed out of the abdomen and collected in 50 µl ice-chilled PBS. To dissect ovaries, females were cooled and cut on the ventral side between second and third pair of legs. The abdomen was then cut laterally and transferred into a drop of PBS. Ovaries were push out of the abdomen and transferred into 50 µl ice-chilled PBS. The qPCR was performed in a 96-well plate format, with a total reaction volume of 15 µl in each well. All samples were further treated as described for D. melanogaster.

**Dscam1 expression in larval D. melanogaster and T. castaneum upon infection.**

To address whether there is a change in Dscam1 expression after bacterial challenge we performed experiments using two different infection routes (e.g., see 10, 11). The first two experiments examined gene expression after haemocoelic bacterial exposure of both D. melanogaster and T. castaneum. In the third we took advantage of the fact that B. thuringiensis can also be orally administered and cause pathogenesis to T. castaneum (1) and therefore examined Dscam1 expression after oral exposure. Infections and dissections were performed blind with respect to treatment as far as possible and treatment orders were randomised within each replicate block.

As a positive control that the immune system had been activated, we additionally tested the expression of three immune genes for each host species. We monitored the expression of **Imd**
and two species-specific antimicrobial peptides (AMPs), *Attacin2* (*Att2*) and *Coleoptericin1* (*Col1*) for *T. castaneum* (12-15) and *Diptericin* (*Dpt*) and *Drosomycin* (*Drs*) for *D. melanogaster* (15-19). In brief, *Imd* plays a signal transduction role in the Imd signalling pathway, this pathway together with the Toll pathway, is responsible for the expression of AMPs after recognition of a bacterial infection (16). *Imd* has been found to be upregulated in *T. castaneum* after infection with some microorganisms (12). In *D. melanogaster* the Imd pathway is activated by Gram-negative bacteria (or Gram-positive bacteria with DAP-type peptidoglycan) and leads to the expression of, e.g., *Dpt*; the Toll pathway is activated by Gram-positive bacteria and fungi and leads to the expression of e.g., *Drs* (19, 20). Both bacteria species we tested, *B. thuringiensis* and *E. coli*, have previously been found to result in increased expression of at least one of our chosen AMPs (e.g., 16, 17). It has been suggested that *T. castaneum* has more promiscuous activation of AMPs than *D. melanogaster* (14), and the AMPs that we chose to test have been shown to be upregulated after infection by bacteria, including *E. coli* (12) *Bacillus subtilis* (14), and another Gram-positive bacteria, *Micrococcus luteus* (12).

**Dscam1 expression after haemocoelic bacterial exposure in larval T. castaneum.**

The experiment consisted of six replicates, each replicate being performed on a different day. Adult beetles were therefore allowed to lay eggs for 24 hour periods over a course of six days. 10 day old *T. castaneum* larvae were individualised in 96-well microtitre plates containing flour and 5 % yeast and incubated as described above. Fifteen day old larvae were pricked dorsolaterally between the second and third segment with a fine needle (2-10 µm Ø) that had previously been dipped into a bacterial suspension or PBS (treatment control, hereafter termed TC), or they were left untreated (naïve control, hereafter termed Naïve). The bacteria concentrations used were: *B. thuringiensis*: $1 \times 10^{10}$ mL$^{-1}$; *E. coli*: $1 \times 10^{10}$ mL$^{-1}$; *P. fluorescens*: $2 \times 10^{7}$ mL$^{-1}$ all suspended in PBS. For this and the following *Dscam1* expression after infection experiments we generally aimed to use bacterial concentrations that resulted in
low mortality (typically between 0 and 20% over three days following exposure). We did not
want to create strong selection for a small sub-group of survivors from which we estimated
gene expression, yet we wanted to elicit an immune response. The bacterial concentration
information was gained from a combination of preliminary experiments and data that we have
previously published. In detail, the *B. thuringiensis* concentration for pricking (1 x 10^{10} cells / 
mL^{-1}) was the same as in (11). Similarly as for *D. melanogaster*, the *E. coli* strain we used is
non-pathogenic for *T. castaneum*, therefore we used the same concentration (1 x 10^{10} cells / 
mL^{-1}) as we used for *D. melanogaster*. The *P. fluorescens* concentration was based upon
preliminary experiments showing that 2 x 10^{7} cells / mL^{-1} resulted in approximately 20% 
mortality at 3 days post exposure. After pricking, the larvae were put into fresh 96-well
microtiter plates containing flour and 5% yeast. The fat body and haemocytes were sampled
six and 18 hours after treatment as described before. We produced six replicates per
combination of treatment / tissue / time point, whereby each replicate consisted of tissue from
ten pooled animals (6 replicates x 5 treatments x 2 tissues x 2 time points x 10 animals = 
1,200 larvae). Survival at six and 18 hours was examined at the time of sampling and we also
produced an extra 14 animals per replicate / treatment / tissue / time point to estimate survival
at 6, 18 and 24 hours (n = 1,680). Average mortality 6 hrs after pricking was less than 1% for
all five treatment groups; at both 18 and 24 hrs after pricking mortality in the *B. thuringiensis*
pricked larvae was ~ 10%, and for all other treatment groups was it was ~ 3% or less.

RNA was extracted, cDNA synthesised and qPCRs run as described in before. For each
sample, *Dscam1* (Table S1) expression was examined as well as the expression of three
immune-related gene: *Attacin2 (Att2), Coleoptericin1 (Col1)* and *Imd* (Table S1). Cp values
were calculated using the Fit Point method with a Noise Band threshold of 3.8844. In some
cases for the infection experiments, the melting curves fulfilled our quality criteria (i.e., were
above detection limit and had a single clear peak at the expected melting temperature), but the
standard deviation (SD) of the Cp value across the two technical replicates was ≥ 0.8. If after
repeating the qPCR the SD was still $\geq 0.8$, or if there was no mRNA signal at all (e.g., *T. castaneum*, *Attacin2*, 18 hour haemocyte sample) we removed the sample (gene/tissue/time point/treatment combination). For *D. melanogaster* this was the case for 38 out of 576 samples, and for *T. castaneum* this was the case for 36 out of 720 samples. These removals explain why in Figure 1 there are sometimes less than 6 biological replicates. Of the remaining samples, in some cases the melting curve fell below the detection limit of the LightCycler®480 software. In these cases we re-did the qPCR. If after the re-run the melting curve was still below the detection limit we used one technical qPCR replicate for that biological replicate where the melting curve of the remaining technical replicate met our quality criteria. This was the case for 19 of the remaining 538 samples for *D. melanogaster* and 16 of the remaining 684 samples for *T. castaneum*. We analysed the data using REST© 2009 (relative expression software tool; 21) and compared the gene expression of two groups at a time: in each case the naïve control group was tested against the treatment control or one of the bacteria-exposed groups. REST calculates the relative fold expression differences by using the expression of reference genes (*Rpl13a* and *Rp49*) to normalise the expression levels of the target genes (*Dscam1*, *Att2*, *Col1* and *Imd*), whilst taking the reaction efficiency (E) of the PCR into account; it is based on the following formula (21, 22):

$$\text{Relative expression} = \frac{E_{\text{target}}^{\Delta C_p \text{ target (control - sample)}}}{E_{\text{reference}}^{\Delta C_p \text{ reference (control - sample)}}}$$

A pair wise fixed reallocation randomisation test© is performed to examine whether there are significant differences between the two groups. We allowed 2000 random reallocations of the observed Cp values to the two groups being tested; REST© notes the expression ratio change for each reallocation, and the proportion of these effects gives the p-value assuming a 2-sided test. In our figures we present the mean and standard errors as calculated according to the REST© software, i.e., the results of the 2000 random reallocations.
Dscam1 expression after haemocoelic bacterial exposure in larval D. melanogaster.

The experiment consisted of six replicates, each replicate being performed on a different day. Four to seven days post-eclosion, flies were allowed to lay eggs on agar plates, as described before. Eggs were collected in the morning and evening for six days, and adults were replaced every two days. Prior to bacteria challenge, late second instar D. melanogaster larvae were removed from the food vials and briefly rinsed in Drosophila Ringer solution and gently dried on a paper towel. Glass capillaries (Hilgenberg GmbH, Germany) that had been pulled to a fine point with a dual-stage glass micropipette puller (PC-10 Narishige) were filled with the bacteria or control solutions and injected into the larvae with a FemtoJet microinjector (Eppendorf AG, Germany). The capillary was inserted laterally towards the posterior end of the larvae at approximately a 20° angle. Bacteria suspensions were prepared in Drosophila Ringer’s solution, diluted 1:10 with a sterile filtered bromophenol blue solution (0.5 mg/mL), such that the final bacterial concentrations were: B. thuringiensis: 7.5 x 10^6 mL^-1; E. coli: 1 x 10^{10} mL^-1. The strain of E. coli that we used is non-pathogenic to D. melanogaster, therefore we could use a relatively high concentration (1 x 10^{10} cells/mL^-1) of bacteria to elicit an immune response resulting in low mortality (~ 95%, 3 days post-injection). B. thuringiensis is more pathogenic than E. coli, and preliminary experiments showed that 1 x 10^7 cells/mL^-1 resulted in 20-30 % mortality 3 days post-infection, and 1 x 10^6 cells/mL^-1 resulted in low mortality, therefore we chose the concentration of 7.5 x 10^6 cells/mL^-1. Bromophenol blue was used to check the injection success, since it is not toxic when fed to larvae (e.g., 23) and it did not induce mortality of larvae after injection and did not negatively affect bacteria survival (personal observation). We produced six replicates per combination of treatment / tissue / time point, whereby each replicate consisted of tissue from ten pooled animals (6 replicates x 4 treatments x 2 tissues x 2 time points x 10 animals = 960 larvae). After injection the larvae were placed individually into 0.2 mL PCR reaction tubes containing fly food, recipe as described above. Survival at 6 and 18 hours was examined at the time of
sampling and we also produced an extra 7 or 8 animals per replicate / treatment / tissue / time point to estimate survival at six, 18 and 24 hours (n = 725). Average mortality 6 and 18 hrs after pricking was less than 2 % for all five treatment groups; at 24 hrs after injection mortality in the B. thuringiensis exposed larvae was less than 4 %, and for all other treatment groups it was it was 0 %. The methods for RNA extraction, cDNA synthesis and qPCR were the same as for T. castaneum except that the immune genes examined were: Diptericin (Dpt), Drosomycin (Drs) and Imd (Table S1). Relative fold expression calculation and statistical analysis was done as described for T. castaneum.

**Dscam1 expression after oral infection in larval T. castaneum.**
The experiment consisted of six replicates, with two replicates being performed per day. Adult beetles were therefore allowed to lay eggs for 24 hour periods over a course of three days. Seventeen day old T. castaneum larvae were exposed to B. thuringiensis spore-containing diet or to a control diet. The B. thuringiensis spore-containing diet was prepared as described in (1) and using the same concentration. In brief, the spore concentration was adjusted to 1 x 10^9 mL^-1 with PBS, and 0.15 g of flour with 5 % yeast was added per mL of spores. Forty microlitres of liquid diet per well was pipetted in a 96 well plate; the plates were covered with breathable sealing foil for culture plates (Kisker Biotech) and placed into individual plastic boxes (Curver, New Grand Chef, 2.6 L) with holes in the lids, which were plugged with foam stoppers to allow for air circulation. To dry the diet the boxes were placed at 50 °C for approx. 17 hours. The diet for the control larvae was prepared in the same way, except flour with yeast was mixed with only PBS. Larvae were exposed to spore-containing or naïve diet for three hours and subsequently transferred to 96-well plates containing the diet without spores, at which point the survival was also monitored. Gut samples were taken six and 18 hours after the initial exposure. To do this, larvae were ice anesthesised, the first and the last segments were removed using a scalpel, and a drop of PBS was added to the sample. The gut was carefully pulled out with a pair of forceps and the fat body was removed. The
guts were washed in a droplet of clean PBS, and ten guts were pooled in 1.5 mL centrifuge tubes containing 50 µL of ice-chilled PBS and the tube was frozen in liquid nitrogen. We produced six replicates per combination of treatment and time point, whereby each replicate consisted of ten pooled animals (6 replicates x 2 treatments x 2 time points x 10 animals = 240 larvae). In addition to monitoring survival at the point of transfer from the spore-containing to the spore-free diet, survival at six, 18 and 24 hours was monitored from additional larvae (n = minimum 480). No larvae had died by 3 or 6 hours after exposure, but at 18 hours 5.1 % B. thuringiensis-exposed and 0.3 % control larvae had died; additional mortality was noted at 24 hours (total ~ 8 %) in the B. thuringiensis group, but there was no further mortality in the control group. Before RNA extraction, the guts were homogenized over liquid nitrogen with a pestle. Further RNA isolation, cDNA synthesis and qPCR was done as described in before, with the addition of immune gene expression (Att2, Coll, and Imd). Cp values were calculated using Fit Point method with a Noise Band threshold of 6.

**Effect of Dscam1 knockdown on larval T. castaneum survival and development after haemocoelic or oral bacterial exposure.**

To address whether there is an effect of Dscam1 knockdown on survival and developmental time we performed two experiments using different infection routes. The first tested survival and development after haemocoelic bacterial exposure and the second tested survival after oral spore exposure.

**Effect of Dscam1 knockdown on larval T. castaneum survival and development after haemocoelic bacterial exposure.**

RNAi is a powerful and well established molecular technique in this species (see 13, 24). To perform knockdown of Dscam1 in T. castaneum larvae we followed the protocol of Posnien et al. (25). A non-alternatively spliced region within the Dscam1 gene, exon 15, was used for RNAi (Figure S1, Table S1; See end of this file for the annotated T. castaneum Dscam1.
gene), which did not overlap with the Dscam1 qPCR primer pair. A gene-specific primer pair for Dscam1 exon 15 (D-ex15) was used to amplify the region of interest from whole body (larval) cDNA. Briefly, RNA extraction and cDNA was produced as described above. To amplify the region of interest we used 1 µL of template cDNA, 10 µL 5x Green GoTaq Flexi Buffer, 26.75 µL water, 5 µL MgCl2 (2.5 mM), 5 µL dNTPs (0.2 mM), 1 µL primers (250 nM) and 0.25 µL GoTaq polymerase (0.5 U) (Promega) and standard PCR conditions (Denaturation: 95°C 2 min; followed by 34 cycles of denaturation: 95 °C 30 sec, annealing: 60 °C 40 sec, and extension: 72 °C 40 sec; final extension: 72 °C 5 min, finally hold at 12 °C). As a treatment control (TCRNAi) for the dsRNA injection we used a 304 bp fragment of the E. coli BL21 (DE3) (Invitrogen) gene asparagine synthetase A (AsnA; gene accession number ECK3738) The maximum sequence similarity of the fragment with the T. castaneum mRNA database is a 16 bp overlap with XM_964818.3 (PREDICTED: Tribolium castaneum death-inducer obliterator 1) which is not sufficient to trigger an RNAi effect (26). The TC construct was synthesized in the same way as the knockdown constructs for Dscam1 with the exception that the gene of interest was directly amplified from a standard colony PCR where we used the following settings: 1 µL of a 1:100 dilution (PBS) from a E. coli colony, 10 µL 5x Green GoTaq Flexi Buffer, 26.75 µL water, 5 µL MgCl2 (2.5 mM), 5 µL dNTPs (0.2 mM), 1 µL primers (250 nM) and 0.25 µL GoTaq polymerase (0.5 U) were mixed and standard PCR conditions (Denaturation: 95°C 10 min; followed by 34 cycles of denaturation: 95 °C 30 sec, annealing: 56 °C 40 sec, and extension: 72 °C 40 sec; final extension: 72 °C 5 min, finally hold at 12 °C) were used. The resulting fragments were cloned into EcoRV linearized pZErO-2™ vector (Invitrogen) according to manufacturer instructions. Constructs pZErO-D-ex15 and pZErO-TC were used as a template in a PCR with vector-specific primers that flanked the fragment with T7 polymerase promoter sequence at their 5' and 3' end. The PCR product was purified with High Pure PCR Cleanup Micro Kit (Roche Diagnostics GmbH, Mannheim) according to the manufacturer’s instructions, and approximately 500 ng
of purified product was used for *in-vitro* transcription with MEGAscript high yield transcription kit (Ambion) according to the manufacturer’s instructions. Twenty microlitres of reaction mix was incubated for five hours at 37 °C and precipitated with LiCl (25 µl LiCl, 30 µl H2O) for one hour at -20 °C. After centrifugation (10,000 rcf, 30 min, 4 °C) and washing the pellet with 70 % EtOH, the pellet was air-dried for 20 to 30 min at RT. The dsRNA was resuspended in PBS and an annealing step was done where the resuspended dsRNA was incubated at 95 °C for two min. Immediately after incubation the dsRNA was incubated in pre-boiled water until the water temperature reached 70 °C, after which followed another incubation at 95 °C in a thermo block. After five min the thermoblock was removed from the heating source and cooled to room temperature. The dsRNA concentration was measured using NanoDrop (NanoPhotometer™ Pearl, Implen) and stored at -80 °C until further use.

To produce the experimental animals, two to three week old adult beetles were allowed to lay eggs for 24 hours. Eleven day old larvae were injected with dsRNA (D-ex15RNAi: n = 256; TCRNAi: n = 256; NRNAi: n = 256) as described in (25). Briefly, the concentration of dsRNA was 2.7 µg/µl for D-ex15RNAi and 2.5 µg/µl for TRNAi. To inject the dsRNA we used glass capillaries that had been pulled with a micropipette puller (as mentioned before). Capillaries were filled with 10 µl dsRNA solution and approximately 300 nl of each dsRNA (ca. 0.8 - 0.9 µg per larvae as recommended by Posien *et al.* (25)) was dorsolaterally injected between the first and second larval abdominal segment using a FemtoJet microinjector. Injected and naïve beetles were individualised into 96-well plates with flour and 5 % yeast. To test the efficiency of the knockdown in the haemocytes and the whole body, we sampled haemocytes from 2 x 10 animals and 2 x 10 whole body larvae from each injection group (D-ex15RNAi and TRNAi) four days after dsRNA injection. *Dscam1* expression was examined for all samples by using qPCR as described before, using *Rp49* and *RpL13a* as reference genes and TC animals as a control group.
Four days after the dsRNA injections, the mortality was as follows: N\textsubscript{RNAi}: 4 %; TC\textsubscript{RNAi}: 10 %; D-ex15\textsubscript{RNAi}: 7 %. Twenty-seven larvae from each group were randomly assigned to one of the following bacteria treatments: \textit{B. thuringiensis} (1x10\textsuperscript{10} cells / mL\textsuperscript{-1}), \textit{B. thuringiensis} (3 x 10\textsuperscript{10} cells / mL\textsuperscript{-1}), \textit{E. coli} (1 x 10\textsuperscript{11} cells / mL\textsuperscript{-1}), wounding control (TC) or naïve (N) groups. The \textit{B. thuringiensis} concentration for pricking was 1 x 10\textsuperscript{10} (as in 11) and 3 x 10\textsuperscript{10} cells / mL\textsuperscript{-1} to increase mortality and potentially any differences between the knockdown treatments. The \textit{E. coli} concentration was 1 x 10\textsuperscript{11} cells / mL\textsuperscript{-1} as used in (27). Survival and development were monitored for seven consecutive days with an additional check fourteen days post infection; by this later check only two larvae (one \textit{E. coli}-challenged D-ex15\textsubscript{RNAi} and one \textit{E. coli}-challenge TC\textsubscript{RNAi}) had died since the seven day check. Larval survival over seven days following haemocoelic bacterial exposure was analysed using the R statistical package (RStudio version 0.99.441) for Macintosh. Within R we used mixed-effects Cox models (packages required: ‘coxme’ (28), ‘survival’ (29, 30), ‘nlme’ (31), ‘bdsmatrix’ (32) and ‘Matrix’ (33)), which allows for the inclusion of random effects. The model was fitted with day of death as the response variable: larvae that were alive at the end of the experiment were included as censored cases. The fixed effects were the knockdown treatment, the infection treatment and the interaction term between these two. Plate was included as a random factor, however it was removed during stepwise elimination of non-significant terms and the final model with only the fixed effects was tested using Cox proportional hazards. There were three groups where no larvae died (TC\textsubscript{RNAi} – \textit{E. coli}, D-ex15\textsubscript{RNAi} – naïve, D-ex15\textsubscript{RNAi} – TC (knockdown – infection treatment)); because there was no event, there was no contribution to the likelihood, and a Cox proportional hazards model could not be fitted. Therefore we denoted one larva as dead in each of these groups allowing us to fit the model. Because the full Cox proportional hazards model did not fulfil the assumptions of proportional hazards over time (based on Schoenfeld residuals) we also tested the model using the survival regression (survreg) function in R. The results from the Cox proportional hazards and survival
regression did not qualitatively differ. Figures are presented without the additional dead larvae. Development time to pupa and adult was analysed using JMP version 9.0.0 for Macintosh OS X. One Dscam1 – E. coli individual remained as a pupa over the course of the experiment so we removed it from the analyses. When individuals died, they died as larvae, except for one D-ex15RNAi – E. coli individual, which died as a pupa. The data could not be transformed to a normal distribution so we performed non-parametric statistics. First we examined whether there was a significant effect of plate on the time to become a pupa or an adult. There was no significant effect of plate on the time to become either a pupa or an adult (pupa: Chi-square = 10.19, df = 7, p = 0.178; adult: Chi-square = 11.28, df = 7, p = 0.127).

The data set was then split by infection treatment and we separately tested, within infection treatment, whether there was an effect of the knockdown on the time to become a pupa or an adult. The only case in which the developmental speed was affected by the knockdown was the time after the infection treatment that it took the uninjected naïve control (NRNAi) animals to become adults (Chi-square = 6.20, df = 2, p = 0.045), where the treatment control for the RNAi (TCRNAi) animals took significantly longer than the NRNAi ones to develop (z = -2.51, p = 0.0226; mean development ± 1 s.e. NRNAi = 11.7 ± 0.23 days, TCRNAi = 12.8 ± 0.39 days, D-ex15RNAi = 13 ± 0.56 days).

Effect of Dscam1 knockdown on larval T. castaneum survival after oral bacterial exposure.

To test survival after oral infection with B. thuringiensis in Dscam1 knockdown T. castaneum larvae, we injected 11 day old larvae with dsRNA (D-ex15RNAi: n = 192; TCRNAi: n = 192; NRNAi: n = 192). The concentration of dsRNA was 2.6 µg/µl for D-ex15RNAi and 2.9 µg/µl for TCRNAi. Injections and preparation of the dsRNA were performed as described before. The efficiency of the knockdown was tested by dissecting three pools of five guts, as described before, and by pooling 3 x 10 whole body larvae from the D-ex15RNAi and TCRNAi injected
groups four days after dsRNA injection. Dscam1 expression was examined for all samples by using qPCR as described before.

Four days after the dsRNA injections we checked larval survival from a subset of injected larvae (mortality: N^{RNAi}: 6 %; TC^{RNAi}: 17 %; D-ex15^{RNAi}: 16 %), and from each injection group 48 size-selected (34) larvae were randomly assigned to a B. thuringiensis spore-containing diet and 48 to a naïve non-spore containing diet. The oral infections were performed as described before with the exception that the spore concentration for this experiment was higher $5 \times 10^9$ mL$^{-1}$, so that we would induce a higher mortality. Larvae were exposed to spore-containing and naïve discs such that 16 larvae from each treatment were assigned to one infection or control 96-well plate. This was replicated three times resulting in a sample size of 48 larvae per treatment group. Survival was monitored for 4 consecutive days. Larval survival after exposure to spores was analysed as described before. None of the larvae from the N^{RNAi}, TC^{RNAi} or D-ex15^{RNAi} groups exposed to only the spore-free diet died; because there was no event, there was no contribution to the likelihood, and a Cox mixed effect model could not be fitted. Therefore we denoted one larva as dead in each of these groups allowing us to fit the model. The fixed effect was knockdown treatment and plate was included as a random factor. However, the latter was removed from the model as it was not statistically significant and the final model with only the fixed effect was tested using Cox proportional hazards.

**Life history effects of Dscam1 knockdown in T. castaneum.**

To examine whether there is a fitness effect, measured via fecundity, and an adult behavioural phenotype after Dscam1 knockdown we performed two simultaneous experiments. We then performed a third experiment to further investigate aspects of fecundity. Unfertilised T. castaneum females will lay eggs (35), therefore we could examine whether knockdown affects egg production even when the mating was unsuccessful. T. castaneum is highly
polygamous – males can mate with up to seven different virgin females in 15 minutes (36).

Males reach sexual maturity approximately two days after imaginal eclosion and females after four days (35); furthermore, copulations are brief, Edvardsson and Arnqvist (37) found that on average they last for around 100 seconds. Therefore they make an ideal model with which to examine questions relating to copulation and mating success.

Experimental animals were produced as described before. For these experiments we produced a second Dscam1 knockdown treatment using exon 12 (D-ex12RNAi; Table S1; Figure S1), the dsRNA was produced as described before. We injected 11 day old larvae with dsRNA (D-ex12RNAi: n = 144; D-ex15RNAi: n = 144; TCRNAi: n = 192; NRNAi (for behavioural assays only): n = 96). The concentration of all dsRNA constructs was 2.7 µg/µl. After injections the larvae were individualised in 96-well plates containing flour and 5 % yeast. After four days, larval survival was checked (mortality: NRNAi: 0 %, TCRNAi: 12 %, D-ex12RNAi: 13 %; D-ex15RNAi: 15 %) and the surviving larvae were transferred into individual glass vials (ø 12.7 mm and 40 mm height with 10 mg of flour plus 5 % yeast) to make it easier to monitor development. Eleven days after the knockdown the pupae were sexed. Twenty-five days post-knockdown the beetles were either used in the behavioural or fecundity assays; only beetles that had been adults for a minimum of three days were used (see 38). Beetles that had not reached the adult stage by 22 days post-knockdown were excluded from the experiment (two individuals from D-ex12RNAi). Mortality between four days post-dsRNA injection and twenty-five days post-dsRNA was as follows: NRNAi: 5 %; TCRNAi: 1 %; D-ex12RNAi: 10 %; D-ex15RNAi: 4 %.

**Behavioural tests.**

Twenty five days post-knockdown we used ten males and ten females from each injection group for the behavioural assays (n total = 80). On the same day, for each of the four treatment groups, we froze three pools of two female and three pools of two male beetles for
later qPCR to check the knockdown (methods as described before), except for D-ex12\textsuperscript{RNAi}, where we froze two pools of females and four pools of males. The assays were performed between 09:30 and 17:00 (daylight hours for the beetle) at room temperature and under light conditions. The assays were carried out in ten blocks, where each block contained one male and one female of each of the treatment groups, i.e. eight beetles, processed in a random order with respect to treatment and sex within each block. We noticed that at the time when beetles were removed from their glass vials for the first behavioural assay that some beetles were positioned ventral side down and others were dorsal side down in the flour, we therefore noted for each beetle whether this was the case or not. When given the opportunity, \textit{T. castaneum} has an innate response to climb. We therefore adapted an assay from Michalczyk \textit{et al.} (39) to test the speed at which beetles climb vertically. The beetles were removed from their glass vials and placed on their backs in individual glass Petri dishes. The beetle was passed blind with respect to treatment to a second experimenter who offered the beetle a white strip of paper (2.5 mm wide x 100 mm long, with a pencil mark at 30 mm), such that the bottom edge of the paper was in contact with the beetle’s tarsi. When the beetle gripped onto the paper, the paper was lifted up from the Petri dish by approximately 10 cm. We measured the time that it took for a beetle to completely pass a 30 mm mark (approximately 10 body lengths), if it managed this it was deemed to have successfully climbed. If the beetle gripped onto the paper and did not climb it was deemed to have successfully gripped (all beetles managed this). If the beetle had not passed the 30 mm mark after one minute the observation was stopped. The beetle was replaced into its original glass vial containing flour and yeast and left at room temperature for 30 min (e.g., as in 40), before a second behavioural test. The glass Petri dishes were wiped with 70\% EtOH between trials (e.g., as in 40).

Behavioural studies in \textit{T. castaneum} have shown that adult beetles are attracted by tall dark shapes (41). By placing the beetles in an open arena surrounded by a dark-coloured wall, we were able to test locomotion ability with respect to the time beetles needed to reach the wall.
To investigate beetle behaviour in an “unprotected environment” (40), the beetle was taken from its glass vial and placed in the centre of an open arena (diameter: 195 mm) surrounded by a dark plastic wall (height: 51 mm). A clean glass vial was placed over the top of the beetle and it was allowed to acclimate for one minute. The glass vial was then removed and the time it took until when the beetle reached the wall (40) was noted; if it reached the wall it was deemed a successful trial. If the beetle had not reached the wall after two min the trial was stopped (unsuccessful trial). In all of the behavioural experiments, except for one, there were groups in which there was no variation in the response variable making it not possible to estimate where significant effects lie. Therefore we only describe these results. It was, however, possible to test whether there was an effect of sex or treatment, or an interaction between the two, on the time that it took the TC\textsuperscript{RNAi} and the N\textsuperscript{RNAi} beetles to reach the wall. The response variable, time, was Box-Cox transformed to achieve a normal distribution within each of the treatment groups. We positioned a Canon EOS 5D Mark II camera directly above the arena and filmed representative beetles from each of the knockdown treatments.

**Fecundity tests.**

On the same day as the behavioural assays we set up female-male pairs in seven different pairings, with twenty pairs per pairing: TC males and females were set up with one another (TC\textsuperscript{RNAi} x TC\textsuperscript{RNAi}) and with males and females of both of the Dscam1 knockdown groups (female first: TC\textsuperscript{RNAi} x D-ex12\textsuperscript{RNAi}; TC\textsuperscript{RNAi} x D-ex15\textsuperscript{RNAi}; D-ex12\textsuperscript{RNAi} x TC\textsuperscript{RNAi}; D-ex15\textsuperscript{RNAi} x TC\textsuperscript{RNAi}), and we also set up males and females from the Dscam1 knockdown treatment with the same treatment (D-ex12\textsuperscript{RNAi} x D-ex12\textsuperscript{RNAi}; D-ex15\textsuperscript{RNAi} x D-ex15\textsuperscript{RNAi}). Because of mortality in the D-ex12\textsuperscript{RNAi} female group, four beetles from the behavioural assay were also used for the fecundity assay. The pairs were kept in plastic vials with 4 g of pre-sieved flour plus 5 % yeast in controlled conditions as described above. The pairs were sieved every three days (e.g., 42) for a total of four sieves. Each time we counted the number of eggs that had been laid and put them back into flour plus 5 % yeast. Female survival was noted and the
pairs were placed in new vials with fresh flour plus 5% yeast. If the male died we allowed the female to continue laying eggs until the end of the experiment. Ten days after each egg count we counted the number of larvae that had hatched. This is the first study to use this wild type stock population, Cro1, in mating assays, and as such we note that the hatching rate of eggs (~80%) estimated from control (TC\textsuperscript{RNAi} x TC\textsuperscript{RNAi}) pairings is comparable to wild type T. castaneum hatching rates that have been found in other populations (35, 43). Egg cannibalism is known to occur in adult T. castaneum (e.g., 35, 44) therefore we cannot exclude the possibility that it might have occurred during the 12-day adult pairing period and affected some of our egg counts. We removed the eggs from the adult pair every three days (the same frequency was used for fecundity analyses by, e.g., 42), meaning that the resulting cohorts of developing larvae were synchronised, which is important because older larvae have been found to have higher egg cannibalism rates than younger larvae (45).

Female survival was analysed as described above, using Cox proportional hazards. The model was fitted with day of death as the response variable. The fixed effect was the combination of the female and male knockdown treatments giving a seven-level factor. There were no female deaths in the TC\textsuperscript{RNAi} x D-ex12\textsuperscript{RNAi} group, therefore for the same rationale as given above, one female was denoted dead in order to run the model. Because some beetles died during the course of the fecundity experiment, to analyse the total number of eggs over the four sieves we excluded any pairs where either the female or the male had died; this resulted in the following numbers of pairs being included in the analysis: TC\textsuperscript{RNAi} x TC\textsuperscript{RNAi} = 18; TC\textsuperscript{RNAi} x D-ex12\textsuperscript{RNAi} = 17; TC\textsuperscript{RNAi} x D-ex15\textsuperscript{RNAi} = 17; ; D-ex12\textsuperscript{RNAi} x TC\textsuperscript{RNAi} = 7; D-ex12\textsuperscript{RNAi} x D-ex12\textsuperscript{RNAi} = 10; D-ex15\textsuperscript{RNAi} x TC\textsuperscript{RNAi} = 6; D-ex15\textsuperscript{RNAi} x D-ex15\textsuperscript{RNAi} = 11. Only nine (26%) of the females from either of the Dscam1 (D-ex12\textsuperscript{RNAi}, D-ex15\textsuperscript{RNAi}) knockdown groups laid eggs, we therefore only tested whether the TC females differed significantly in the number of eggs that they laid depending upon who they had been paired with. The data were normally distributed and had equal variances so we performed an ANOVA using JMP. Apart
from two D-ex12RNAi knockdown females paired with TC males, larvae only hatched from the pairings between TCRNAi treated females and males, we therefore did not statistically analyse this data set.

**Mating behaviour and physiology.**

In the fecundity experiment above the females and males were placed together continuously and no observations were made of mating behaviour. In this experiment we therefore aimed to examine whether knockdown females and males mate and also whether female knockdown beetles show evidence of reduced ovaries. Experimental animals were produced as described before. We injected 11 day old larvae with dsRNA (D-ex12RNAi: n = 72; D-ex15RNAi: n = 72; TCRNAi: n = 72; NRNAi: n = 72). The concentration of dsRNA constructs was 2.4 µg/µl for D-ex12RNAi and D-ex15RNAi, and 2 µg/µl for TCRNAi. After injections the larvae were individualised in 96-well plates containing flour and 5 % yeast. After four days, larval survival was checked (mortality: NRNAi: 1 %, TCRNAi: 21 %, D-ex12RNAi: 10 %, D-ex15RNAi: 14 %) and the surviving larvae were transferred into individual glass vials (ø 12.7 mm and 40 mm height with 10 mg of flour plus 5 % yeast) to make it easier to monitor development. Eleven days after the knockdown the pupae were sexed. Twenty-eight days post-knockdown the beetles were assayed; only beetles that had been adults for a minimum of six days were used. Mortality between four days post-dsRNA injection and twenty-eight days post ds-RNA was as follows: NRNAi: 8 %, TCRNAi: 20 %, D-ex12RNAi: 47 %, D-ex15RNAi: 25 %.

We set up females and males in ten different pairing combinations. As a full control, we paired TCRNAi x TCRNAi. We additionally included NRNAi x NRNAi. We also set up all combinations of TCRNAi and NRNAi females and males with both knockdown females and males, i.e., female first: TCRNAi x D-ex12RNAi; TCRNAi x D-ex15RNAi; D-ex12RNAi x TCRNAi; D-ex15RNAi x TCRNAi; NRNAi x D-ex12RNAi; NRNAi x D-ex15RNAi; D-ex12RNAi x NRNAi; D-ex15RNAi x NRNAi. Because in the previous experiment D-ex12RNAi x D-ex12RNAi and D-ex15RNAi x D-
ex15RNAi did not produce eggs we did not include these combinations. All pairings were set up in five replicates, except for TCRNAi x TCRNAi and TCRNAi x D-ex15RNAi where we had four replicates each. The assays were performed between 11:00 and 18:00 (daylight hours for the beetle) at room temperature and under light conditions. In a previous experiment we had observed that some knockdown females had an everted ovipositor, this phenotype has also since been detailed on ibeetle (46), therefore prior to pairing we checked all females and males under a dissecting microscope for everted genitalia. Females were allowed four minutes to acclimatise to a plastic Petri dish (3.5 cm diameter), where the bottom surface had been thoroughly scratched to provide a rough surface for the beetles to right themselves if they fell over. After four minutes we added the male and noted the time at which he had been added. We noted the time at which the male appeared to start copulating with the female (when he mounted her and it looked as if genital contact had been made), and also the time at which the pairing ended. An attempted copulation was defined as an interaction where genital contact appeared to be maintained for at least 35 seconds: Edvardsson & Arnqvist (37) found that male T. castaneum with a copulation duration of shorter than 36 seconds did not father any offspring. The mating durations that we found in this experiment for our wild-type (mean duration in seconds ± 1 S.E.: NRNAi x NRNAi = 207 ± 113; TCRNAi x TCRNAi = 134 ± 13) are comparable to previous findings from other wild-type T. castaneum populations e.g. (37, 47). If directly after copulation the male had no immediate further interest in the female we separated the pair. However, if the pair separated and the male immediately had further interest in the female, by attempting to mount her and rubbing the lateral edges of her elytra with his tarsi (37), we left the pair together. In these cases where there were multiple attempts by the male to mate the female, we observed them until there was a 10 minute period with no interaction and then separated them. With the exception of one TCRNAi x TCRNAi pairing that had two mating attempts, multiple attempts to mate always involved a knockdown female paired with a TCRNAi or NRNAi male. The pairs were given a maximum of one hour in which to
mate, after this time they were separated and females were placed in individual plastic vials containing 4 g of pre-sieved flour plus 5 % yeast and kept in controlled conditions as described above. The flour was sieved after three days and the number of eggs was counted. The eggs were kept for ten days and after this time the numbers of larvae were counted. The ovaries of twenty females were dissected on the same day that the eggs were counted; they were then photographed with a Canon EOS 5D Mark II under a dissecting microscope. We present the data from this experiment as descriptive because the responses measured were mostly binary and because of the relatively low sample sizes.

On the same day as the pairs were set up, five males and five females from each of the four treatment groups were placed individually into a thin glass tube (100 mm x 5 mm) with a strip of paper (width 3.5 mm) lining the bottom. The last 30 mm of the tube were covered with a piece of black cotton. We positioned a Canon EOS 5D Mark II camera with a macro lens to the side of the glass tube to film the beetles, thus providing some lateral footage of the beetles’ movements as they walked, and we left the beetles inside the tube for a maximum of one minute and noted whether or not they reached the darkened area of the tube.
| Exon | Primer Forward | Primer Reverse |
|------|----------------|----------------|
| 1    | 1              | 5              |
| 2    | 2              | 5              |
| 12   | 12             | Primer Reverse |

Comment [9]: Exon 11

Comment [10]: Start beetle RNAi construct

Length: 510 bp

For more details see:

http://ibeetle.uni-goettingen.de/details/db_05264

Comment [11]: ePCR Primer Forward

Use Table S1 for more detail

Comment [12]: Dscam1

See Table S1 for more detail

Comment [13]: Dscam1

See Table S1 for more detail

Comment [14]: Dscam1

See Table S1 for more detail

Comment [15]: Exon 12

174 bp

Comment [16]: End beetle RNAi construct

Comment [17]: Exon 13

1059 bp

Comment [18]: Exon 14

166 bp

Comment [19]: Dscam1

See Table S1 for more detail

Comment [20]: Dscam1

See Table S1 for more detail

Comment [21]: Dscam1

See Table S1 for more detail

Comment [22]: Exon 16

503 bp

Comment [23]: Exon 17

130 bp

Comment [24]: Exon 18

167 bp

Comment [25]: Exon 19

159 bp

Ortholog to exon 17.1 in D mel
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