Supplementary materials for:

Social cues trigger differential immune investment strategies in a non-social insect, *Tenebrio molitor*

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Supplementary methods are described below in sections S1-S4. Supplementary data, including full model outputs, are described below in sections S5 and S6.

Supplementary methods

S1. Bacterial preparation and preliminary dose response survival analyses

As a pathogen challenge, we used erythromycin-resistant *Staphylococcus aureus* (strain SH1000). This is a Gram-positive bacterium found in tenebrionid food stores [1] which causes persistent infection and induces an immune response in *T. molitor* that extends over the 72 h cohabitation period [2] used in our experimental design (described in section S2). In order to provide a non-pathogenic immune elicitor, bacterial heat-killing was performed by heating inoculates in a water bath at 95°C for 30 mins. Efficacy of the heat-killing treatment was confirmed by plating onto standard agar and incubating at 37°C for 24 hours, which yielded zero colonies in total (n = 6).

A survival analysis estimated an LD_{50} after 14 days at 5.33 x 10^7 colony forming units (CFUs). The effect of food availability on survival in bacterially-infected groups was significant (Cox proportional hazards: HR = 1.64, WS = 9.23, N = 178, d.f. = 1, P = 0.002; Fig. S1).

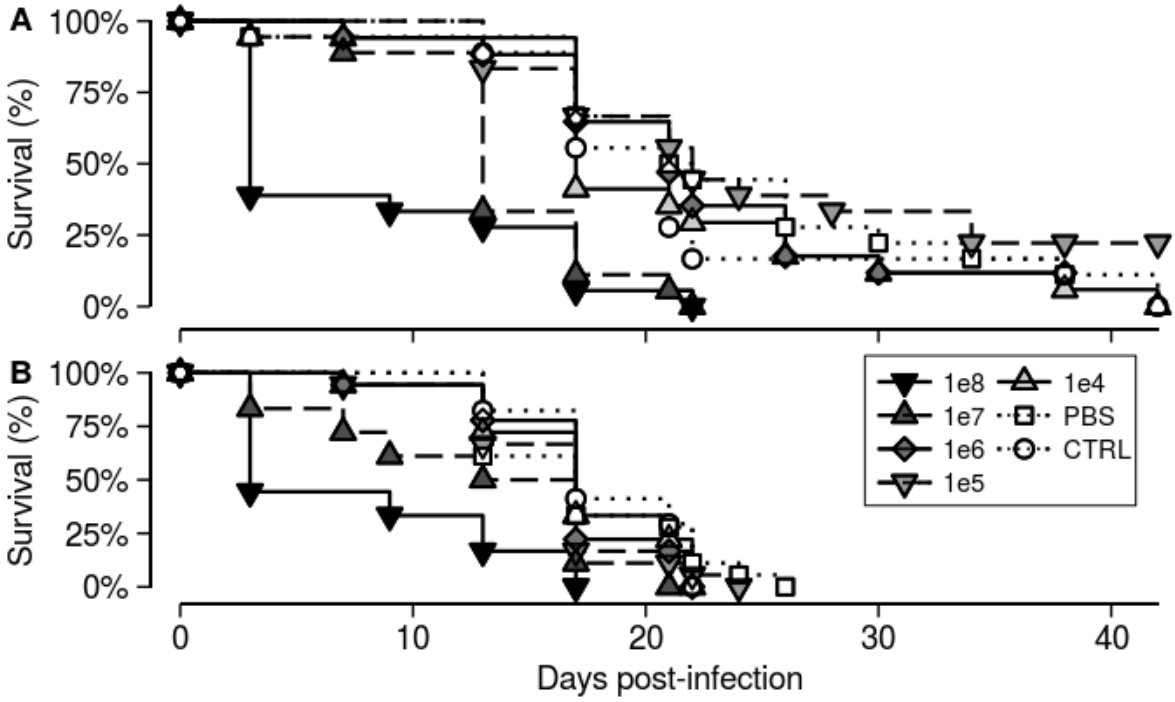

Figure S1. Survival of non-cohabiting adult females after injection with live *S. aureus* under (**A**) *ad libitum* feeding conditions or (**B**) starvation. Symbols and lines depict the injection treatment.

Cohabitants in Experiment 1 (fig. S2a) were injected with 2.5 x 10^4 CFUs of both live and heat-killed bacteria to ensure that doses were matched, and that they induced a sub-lethal immune response. Focal beetles assayed for antibacterial activity by the haemolymph in Experiment 1 (fig. S2a) were injected with a higher dose of 2.5 x 10^6 CFUs live bacteria to ensure that variation in bacterial clearance by the host could be detected. Similarly, focal beetles assayed for survival were injected with the LD_{50} dose of 5 x 10^7 CFUs in Experiment 2 (fig. S2b); as we were only using heat-killed bacteria, we could afford to use the higher dose of 2.5 x 10^6 CFUs for both cohabitant and focal beetle treatment. This dose was chosen to ensure effective immune stimulation in beetles whilst also limiting impacts on survival, as we were primarily interested in egg production as opposed to survival after infection.

S2. Cohabitant treatment design

Pairs of female beetles were housed together in Petri dishes (50 mm) for 72 h with *ad libitum* access to food and water. Each pair consisted of a naive focal and treated cohabitant, with the cohabitants from Experiment 1 receiving the treatment regime depicted in fig. S2a, and the cohabitants from Experiment 2 receiving the treatment regime depicted in fig. S2b. After 72 h, focal beetles were removed from their pairs and randomly assigned to the sub-treatments specifically designed to test the effect of social immunisation on their resistance and tolerance (Experiment 1, fig. S2a) or fitness (Experiment 2, fig. S2b) traits.

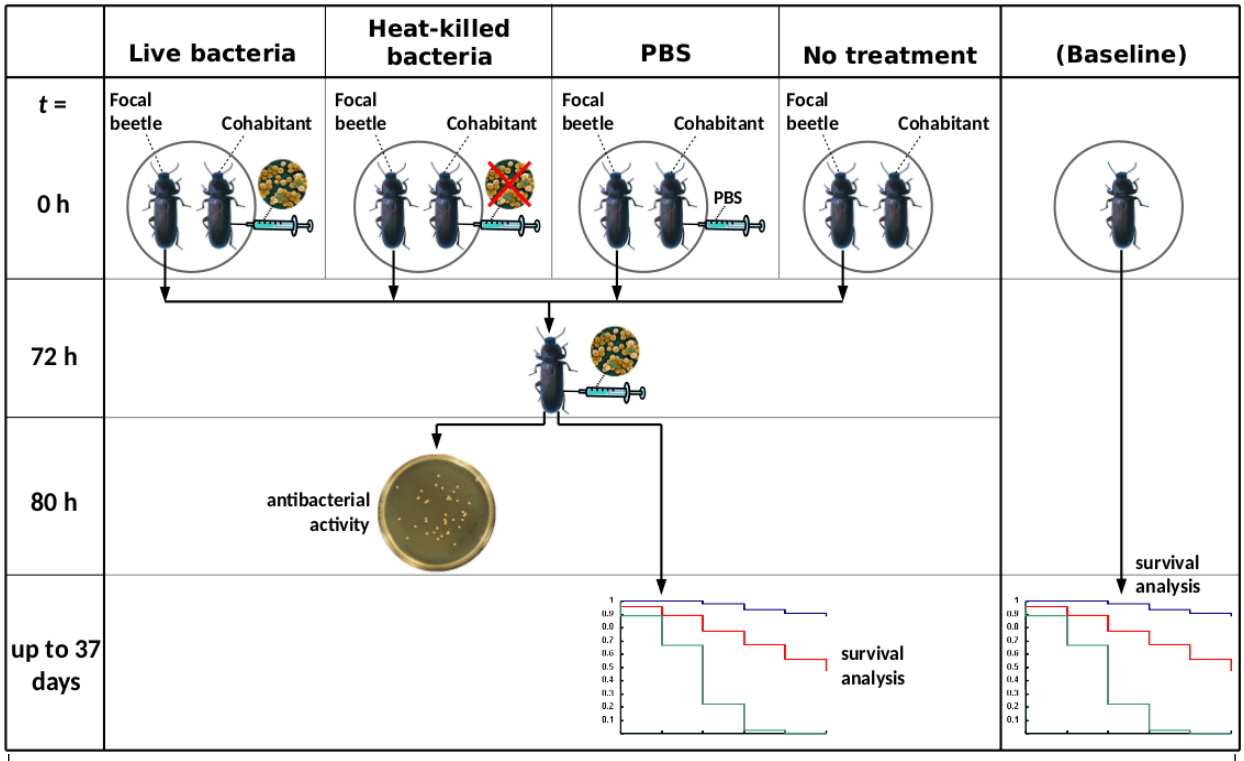

Figure S2a. Experimental design for Experiment 1. Pairs of female beetles were housed together in Petri dishes (diameter 50 mm) for 72 h with *ad libitum* access to food and water. *C*ohabitants received one of the following treatments: 2.5 x 10^4 CFUs of either (i) live or (ii) heat-killed bacteria suspended in 5 µL sterile PBS, (iii) 5 µL sterile PBS as a wounding control, or (iv) no treatment. After 72 h, focal beetles were removed from their pairs and randomly assigned to one of two sub-treatments to measure *in vivo* antibacterial activity (n=76) or survival (n=126) in response to infection. A baseline group (n=32) of untreated, non-cohabiting individuals from the same cohort were maintained as a procedural control.

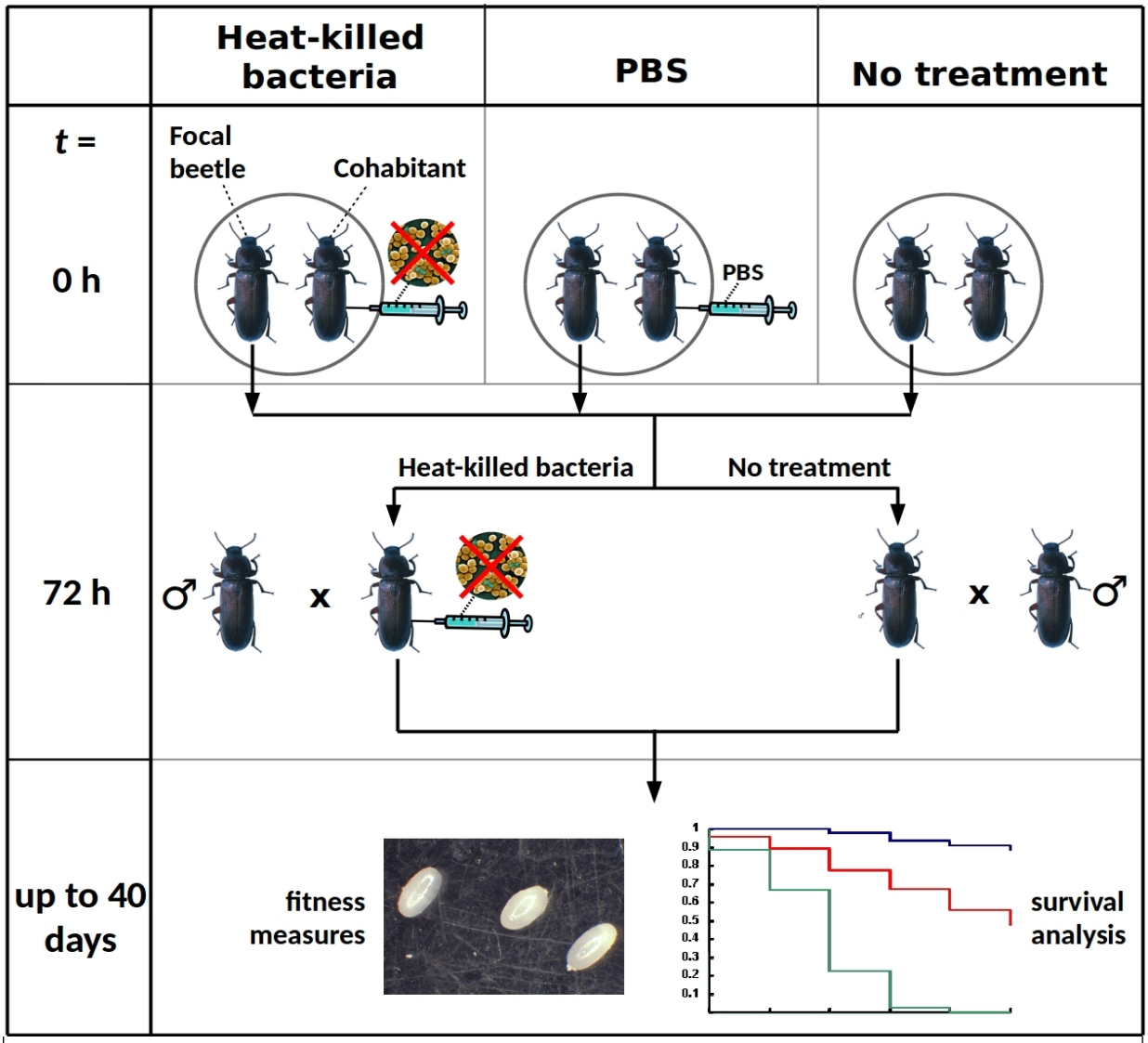

Figure S2b. Experimental design for Experiment 2. Pairs of beetles were housed as in Experiment 1, but cohabitants received one of the following treatments: (i) injection with 2.5 x 10^6 CFUs heat-killed bacteria, (ii) injection with 5 µL sterile PBS, or (iii) no treatment. After 72 h cohabitation, focal beetles were randomly assigned to one of two sub-treatments; one half (n = 224) received the same challenge as the heat-killed bacteria-challenged cohabitants, whilst the other half (n = 224) remained unchallenged. All focal beetles were then mated with an age-matched, unchallenged, virgin male for 24 h before being kept in isolation with *ad libitum* food and water for the next 40 days to monitor survival and take fitness measurements (egg count, egg volume).

S3. Bacterial clearance assay

Perfused haemolymph samples from the focal beetles were serially diluted up to 10^{-4} in sterile PBS, and 200 µL of each dilution was plated out onto erythromycin-infused agar (10 µg mL^{-1} erythromycin and 5.6 µg mL^{-1} amphotericin-B) to selectively grow and recover only the erythromycin-resistant *S. aureus*. Plates were incubated at 37°C for 48 h and CFUs enumerated using OpenCFU [3]; plates with 10-100 CFUs were preferentially used to calculate total residual bacterial load. 99.5% *Staphylococcus aureus* are cleared within 1 h of injection [2] but induced antimicrobial activity is only detected after 6 h and peaks after around 4 days [2]. We perfusion bled (see [4]) focal beetles at 8 h post-infection as there is strong transcription at this time [5] and long-lasting immune defence has been induced at this point [2].

S4. Egg volume calculation

To estimate egg size, three eggs were randomly sampled from each egg count taken from focal beetles, and digitally photographed against a dark background using a MicroPublisher 3.3 RTV camera (QImaging, Burnaby, BC, Canada) attached to a Leica dissecting stereoscope (Wetzlar, Hessen, Germany). An automated image analysis script was developed in C++ using the open-source image analysis library, OpenCV (http://opencv.willowgarage.com) and is available online (https://github.com/JoGall/egg-size). In short, the script: (a) converted each image to greyscale, (b) blurred, (c) adaptive thresholded to identify foreground, (d) identified the three largest contours, (f) fitted a minimum rotated rectangle fitted to each contour, (g) returned the length and width of each rotated rectangle as an estimate of egg length and width, (h) outputted the resulting image mask for validation (fig. S4). Estimates of egg length and width were scaled to true spatial units (mm) using a calibration image. Volume was estimated using the following formula for the area of a regular ellipsoid, where a = egg length and b = egg width / 2 (see [6]):

$$V = \frac{4}{3} \cdot \pi \cdot a \cdot b^2$$

Tenebrionid eggs are covered by a sticky exochorion to which flour particles often adhere [7]; in some cases, this resulted in overestimation of egg length and width due to surrounding flour being identified as part of the egg. Image masks were therefore reviewed and in such instances the raw images edited manually by highlighting problematic areas in black to mark them as background. This resulted in measures of egg length and width which more closely matched estimates by human observers. The reliability of our image analysis method was assessed by comparing with measurements from two human observers across a sample of six images (18 eggs). Automated estimates of egg length and width differed on average by 3.7% and 3.4% (respectively) from one human observer, and by 2.7% and 3.5% from the other.

This is comparable to the difference between the two human observers, where egg length and width differed on average by 2.0% and 3.3%.

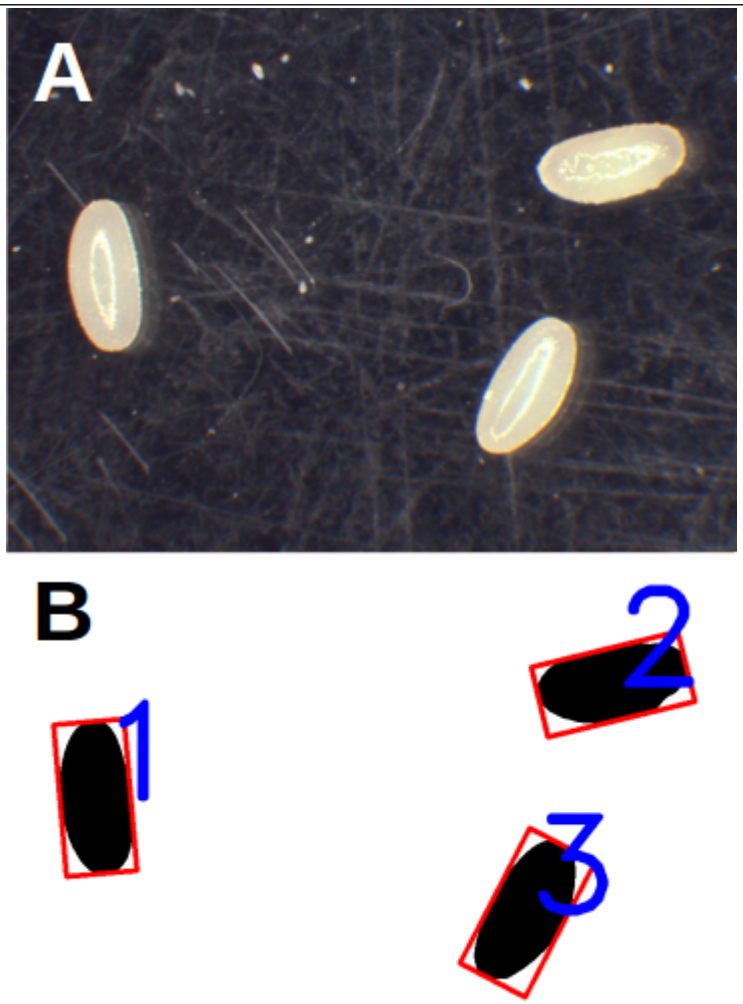

Figure S4. (**A**) Raw image of three eggs from a focal beetle and (**B**) processed mask showing identified foreground (black) and bounding box (red) from which length and width estimates were taken.

Egg volume was measured only when three or more eggs had been laid per beetle per count, meaning that the data set was truncated. However, the level of data truncation between treatments is minimal and even, and the number of data points that egg volume measurements were made on remains large (Table S4.1).

Table S4.1. Data collected during fitness measurements to show extent of data truncation before image processing.

Treatment	**Total # eggs**	**Total # egg counts**	**# egg counts with < 3 eggs**	**% data truncation**
Heat-killed / Heat-killed	613	82	24	29
Heat-killed / No treatment	1065	130	37	28
PBS / Heat-killed	433	70	30	43
PBS / No treatment	907	96	13	13
No treatment / Heat-killed	732	105	33	31
No treatment / No treatment	985	117	31	27

Supplementary data

S5. Full outputs from statistical analyses

Experiment 1 (S5.1 – S5.6)

Table S5.1. Model output from negative binomial regression fitted to antibacterial activity data from focal beetles from Experiment 1. Treatment refers to non-focal beetle treatment and weight refers to focal beetle pupal weight.

Variable	d.f.	F value	P-value
Treatment	3	1.413	0.246
Weight	1	4.414	0.039 *

Table S5.2. Multiple comparison output using Tukey's HSD from negative binomial regression fitted to antibacterial activity data from focal beetles from Experiment 1, including weight as a covariate.

Comparison	z value	P-value
Heat-killed bacteria – No treatment	0.395	0.979
Live bacteria – No treatment	-0.402	0.978
PBS – No treatment	0.298	0.991
Live bacteria – Heat-killed bacteria	-0.813	0.848
PBS – Heat-killed bacteria	-0.078	1.000
PBS – Live bacteria	0.698	0.898

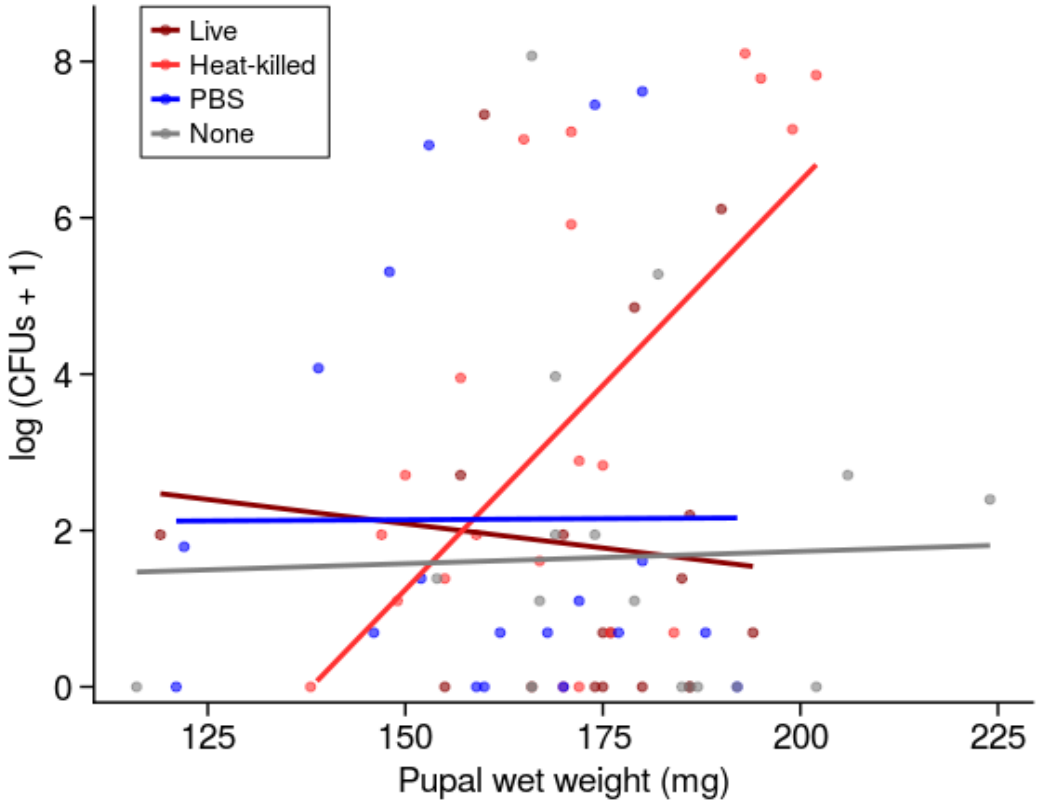

Figure S5.3. Importance of pupal weight in explaining cohabitant beetle treatment effect on antibacterial activity (as measured by the number of *Staphylococcus aureus* colony forming units [CFUs] recovered from their haemolymph 8 h post-infection). Line colour denotes cohabitant treatment.

Table S5.4. Model output from Cox proportional hazards model fitted to data from focal beetles from Experiment 1. Treatment refers to non-focal beetle treatment and weight refers to focal beetle pupal weight.

Variable	**d.f.**	**Chisq value**	**P-value**
Treatment	3, 71	10.488	0.015 *
Weight	1, 71	3.075	0.080

Table S5.5. Survival parameters for focal beetles from Experiment 1 by treatment (survival time in days [mean ± S.E.] and Cox proportional hazard [CPH] ratios [mean ± S.E.] vs. comparison treatment group). CPH regressions showed a significant efffect of post-cohabitation challenge on survival (treatment groups vs. baseline: LR = 106.0; d.f. = 4,158; $p < 0.001$), and a significant effect of cohabitant treatment on survival among (non-baseline) treatment groups (LR = 10.5; d.f. = 3,126; p = 0.015).

	Cohabitant treatment				
Parameter	**Live**	**Heat-killed**	**PBS**	**None**	**(Baseline)**
Survival time	7.7 ± 1.5	11.8 ± 1.8	15.7 ± 2.7	13.8 ± 2.3	35.69 ± 1.09 †
CPH vs. None	1.84 ± 0.26 *	1.27 ± 0.26	0.77 ± 0.28	-	-
CPH vs. PBS	2.37 ± 0.28 **	1.64 ± 0.28	-	1.29 ± 0.28	-
CPH vs. Heat-killed	1.45 ± 0.25	-	0.61 ± 0.28	0.79 ± 0.26	-
CPH vs. Live	-	0.69 ± 0.25	0.42 ± 0.28	0.55 ± 0.26	-
CPH vs. (Baseline)	68.4 ± 0.74	47.4 ± 0.74	29.9 ± 0.74	37.5 ± 0.74	-

* p = 0.09; ** p = 0.01

† Longevity for right-censored data incorporated as 37 days for the purpose of calculating mean survival time

Table S5.6. Multiple comparison output using Tukey's HSD Cox proportional hazards model fitted to data from focal beetles from Experiment 1, excluding weight as a covariate.

Comparison	**z value**	**P-value**
Heat-killed bacteria – No treatment	0.902	0.804
Live bacteria – No treatment	2.326	0.092
PBS – No treatment	-0.935	0.786
Live bacteria – Heat-killed bacteria	-0.935	0.456
PBS – Heat-killed bacteria	-1.776	0.285
PBS – Live bacteria	-3.115	0.0096 **

Experiment 2 (S5.7 – S5.13)

Table S5.7. Model output from linear regression fitted to egg-laying rate data from Experiment 2. Cohabitant and Focal refers to treatment category applied to relevant beetles, weight refers to beetle pupal weight.

Variable	**d.f.**	**F value**	**P-value**
Cohabitant	2, 218	0.066	0.936
Focal	1, 218	14.131	< 0.001 ***
Weight	1, 218	0.721	0.397
Cohabitant*Focal	2, 218	2.332	0.099

Table S5.8. Multiple comparison output using Tukey's HSD linear regression fitted to egg-laying rate data from Experiment 2, excluding weight as a covariate.

Comparison (cohabitant/focal)	**t value**	**P-value**
No treatment/Heat-killed – Heat-killed/Heat-killed	0.374	0.999
PBS/Heat-killed – Heat-killed/Heat-killed	-0.935	0.937
Heat-killed/No treatment – Heat-killed/Heat-killed	1.995	0.348
No treatment/No treatment – Heat-killed/Heat-killed	1.175	0.848
PBS/No treatment – Heat-killed/Heat-killed	2.853	0.053
PBS/Heat-killed – No treatment/Heat-killed	-1.318	0.775
Heat-killed/No treatment – No treatment/Heat-killed	1.648	0.568
No treatment/No treatment – No treatment/Heat-killed	0.812	0.965
PBS/No treatment – No treatment/Heat-killed	2.519	0.123
Heat-killed/No treatment – PBS/Heat-killed	2.916	0.045 *
No treatment/No treatment – PBS/Heat-killed	2.113	0.284
PBS/No treatment – PBS/Heat-killed	3.762	0.003 **
No treatment/No treatment – Heat-killed/No treatment	-0.846	0.958
PBS/No treatment – Heat-killed/No treatment	0.872	0.953
PBS/No treatment – No treatment/No treatment	1.723	0.518

Table S5.9. Model output from linear mixed effects model fitted to egg volume data from Experiment 2. Cohabitant and Focal refers to treatment category applied to relevant beetles, weight refers to beetle pupal weight.

Variable	**d.f.**	**F value**	**P-value**
Cohabitant	2, 416	3.928	0.020 *
Focal	1, 416	17.601	<0.001 ***
Weight	1, 416	4.364	0.003 **
Cohabitant*Focal	2, 416	11.431	<0.001 ***

Table S5.10. Multiple comparison output using Tukey's HSD linear mixed effects model fitted to egg volume data from Experiment 2, including weight as a covariate.

Comparison (cohabitant/focal)	**z value**	**P-value**
No treatment/Heat-killed – Heat-killed/Heat-killed	2.321	0.183
PBS/Heat-killed – Heat-killed/Heat-killed	1.855	0.426
Heat-killed/No treatment – Heat-killed/Heat-killed	6.278	<0.001 ***
No treatment/No treatment – Heat-killed/Heat-killed	3.855	0.002 **
PBS/No treatment – Heat-killed/Heat-killed	1.632	0.573
PBS/Heat-killed – No treatment/Heat-killed	-0.176	0.999
Heat-killed/No treatment – No treatment/Heat-killed	3.959	0.001 **
No treatment/No treatment – No treatment/Heat-killed	0.464	0.684
PBS/No treatment – No treatment/Heat-killed	-0.812	0.965
Heat-killed/No treatment – PBS/Heat-killed	3.515	0.006 **
No treatment/No treatment – PBS/Heat-killed	1.426	0.708
PBS/No treatment – PBS/Heat-killed	-0.512	0.996
No treatment/No treatment – Heat-killed/No treatment	-2.662	0.082
PBS/No treatment – Heat-killed/No treatment	-5.081	<0.001 ***
PBS/No treatment – No treatment/No treatment	-2.427	0.145

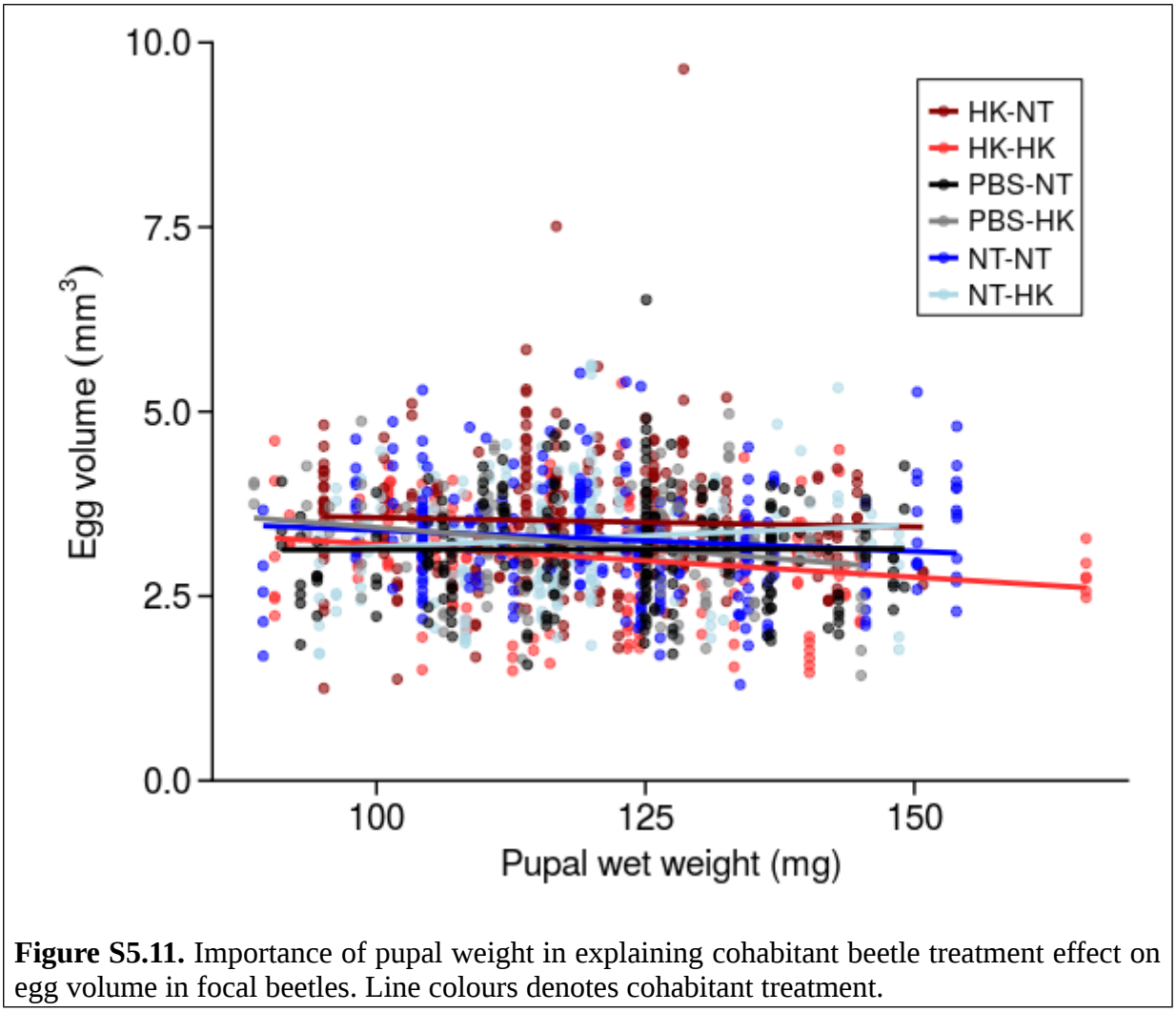

Figure S5.11. Importance of pupal weight in explaining cohabitant beetle treatment effect on egg volume in focal beetles. Line colours denotes cohabitant treatment.

Table S5.12. Survival parameters for focal beetles from Experiment 1 by treatment (survival time in days [mean ± S.E.] and Cox proportional hazard [CPH] ratios [mean ± S.E.] vs. comparison treatment group). Cox proportional hazard regression failed to show a significant effect of nonfocal beetle treatment (LR = 7.34; d.f. = 5,224; p = 0.197).

	Cohabitant treatment – Focal treatment					
Parameter	**NT-NT**	**NT-HK**	**PBS-NT**	**PBS-HK**	**HK-NT**	**HK-HK**
Survival time	18.2 ± 0.9	16.5 ± 0.9	15.4 ± 1.1	15.2 ± 0.61	17.8 ± 1.2	15.3 ± 0.9
CPH	-	1.38 ± 0.23	1.53 ± 0.24	1.66 ± 0. 24	1.10 + 0.24	1.53 ± 0.24

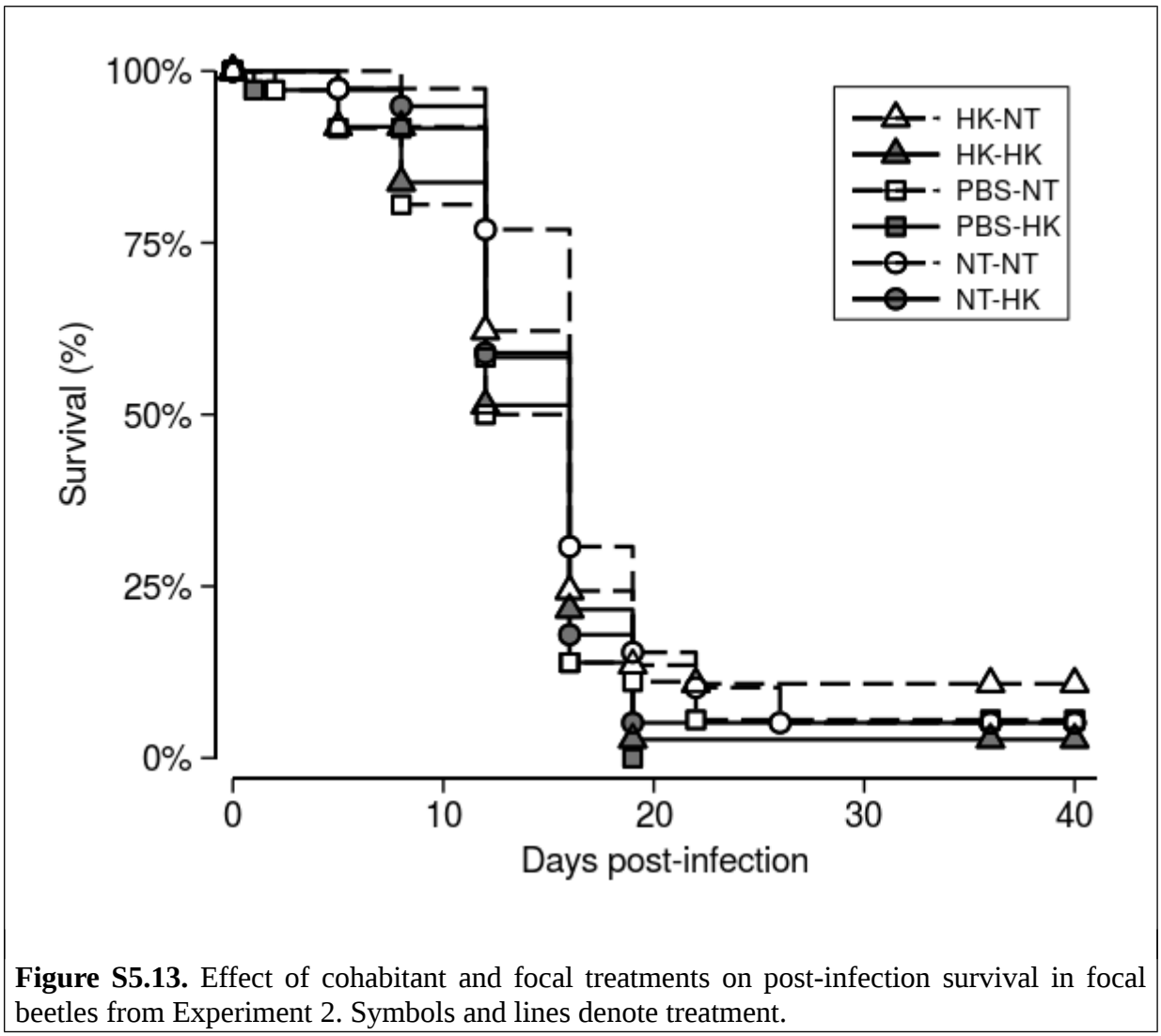

Figure S5.13. Effect of cohabitant and focal treatments on post-infection survival in focal beetles from Experiment 2. Symbols and lines denote treatment.

S6. Extra data and analyses

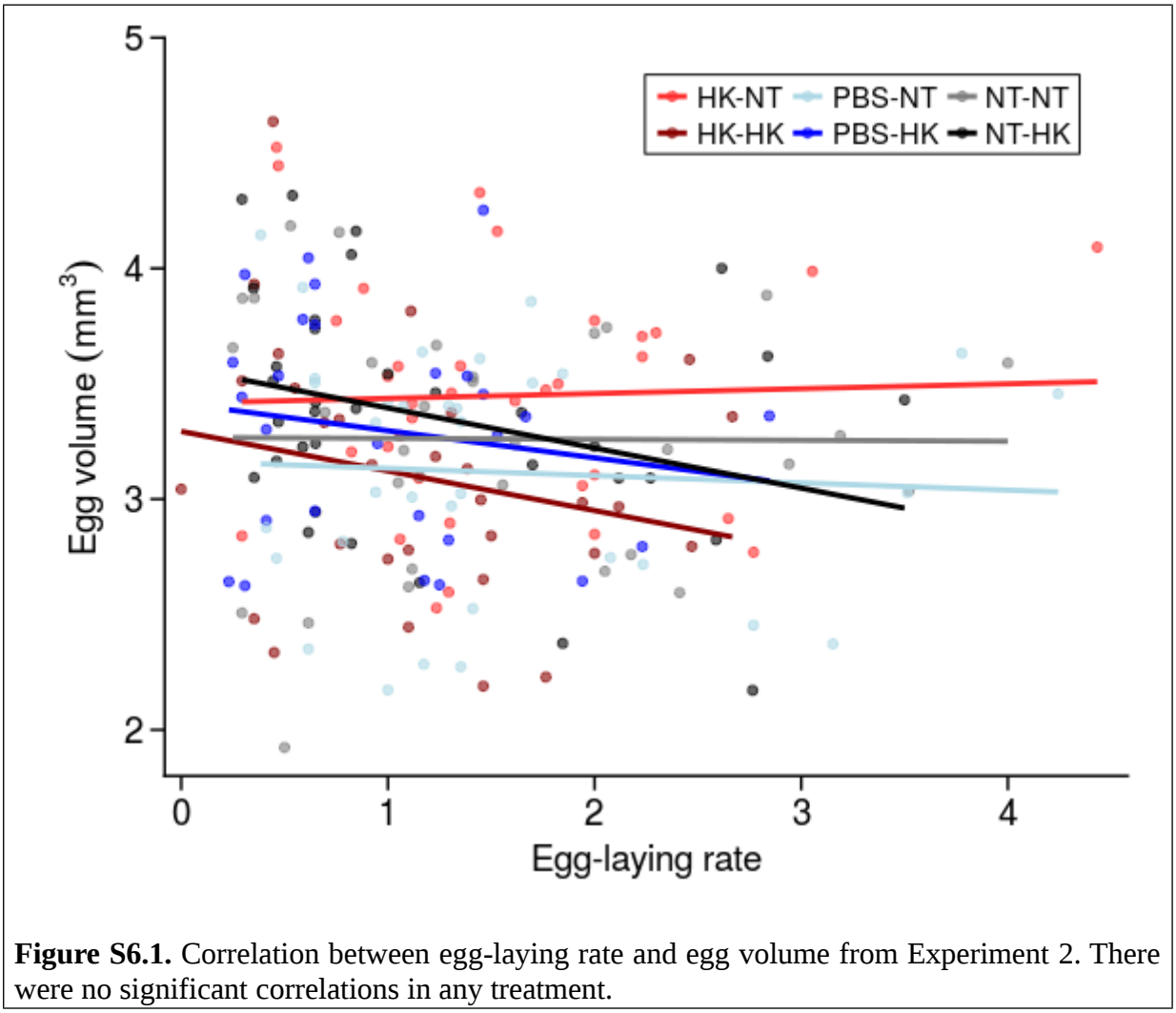

Figure S6.1. Correlation between egg-laying rate and egg volume from Experiment 2. There were no significant correlations in any treatment.

Changes in egg-laying rate and egg volume over time are depicted below, we do not analyse effects of time in our data, because over the 40 day period, the number of laying beetles decreases due to their deaths. The data is truncated and any analyses would be very difficult to interpret, but we include fig. S6.2 for general interest.

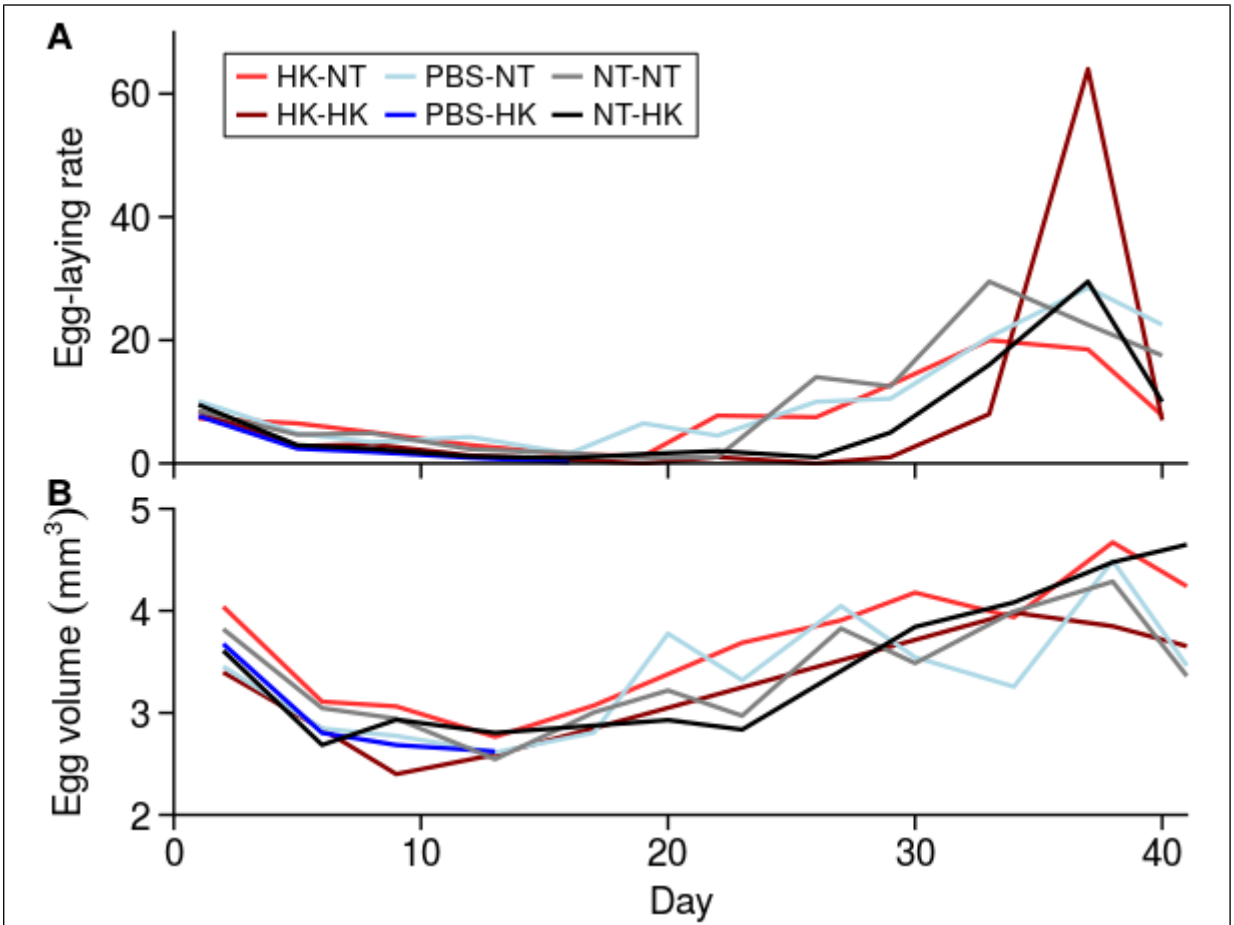

Figure S6.2. (A) Mean egg-laying rate (expressed as total number of eggs produced per day divided by the number of individuals alive) and (**B**) mean egg volume of focal beetles by treatment over the course of Experiment 2. Line colour denotes treatment.

References

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