Electronic Supplementary Material (ESM)

Proceedings of the Royal Society B: Biological Sciences

DOI: 10.1098/rspb.2017-1711

Neonicotinoid pesticides and nutritional stress synergistically reduce survival in honey bees

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Methods

Honey bee preparation

We captured forager bees from the colonies using funnel traps and starved them for 1 h in a bee collection container [1]. This starvation period allowed the bees to reach a more uniform nutritional status, which was facilitated by food exchange among bees within the container. The confinement also increased bee hunger and thereby facilitated the complete and rapid consumption of the sugar solutions that we subsequently provided. We then placed bees into disposable cardboard cages (15 bees per cage) that were 9.5 x 6.5 x 5 cm, had 1 mm diameter holes in the bottom for ventilation, and a transparent acetate front wall for observation. To facilitate handling, we anesthetized the bees with a 40-60% air-CO_2 mixture for 2 min. Although the 2 min CO_2 exposure reduced bee activity, they were not completely paralyzed and could move their abdomens and breathe. Preliminary tests, carried out on foragers from the same apiary, showed that this was the lowest CO_2 concentration that led to successful anesthetization of the bees and resulted in a recovery without side effects. Previous studies have shown that such a brief CO_2 exposure did not influence hemolymph sugar concentrations [2]. We maintained the cages in a dark incubator at 25 ± 1°C and 50-80% RH throughout the duration of the experiment (methods of [3,4]).

Sugar diet treatments

The sugar content of nectar depends upon various factors, including plant species and variety [5]. Because bees can discriminate a 5% difference in sugar concentrations [5], we exposed bees to sugar treatments with a higher difference (17.5%) in sugar content.

The *ad libitum* diet was provided in a 2.5 mL syringe suspended inside the cage and renewed daily for the entire experiment (4 days). The *limited* quantity diet consisted of 10 µL/bee total, and was provided inside an Eppendorf centrifuge tube cap placed inside the cage [3,4]. In the *no nutrients* treatment, each cage received a *limited* diet consisting of distilled water only (three repetitions with three replicates each). The diets were prepared with analytical grade sucrose, double-distilled water, and, in pesticide treatments, analytical grade TMX or CLO.

Neonicotinoid treatments

We tested sublethal acute oral exposure to field-realistic doses of two neonicotinoid pesticides: thiamethoxam (TMX, CAS# 153719-23-4, Dr. Ehrenstorfer GmbH) and clothianidin (CLO, CAS# 205510-92-5, Dr. Ehrenstorfer GmbH). We followed the most recent international guidelines for pesticide tests on bees [4], and provided 150 µL (corresponding to 10 µL/bee) of pesticide test sugar solution to each cage in Eppendorf caps. In all cages, bees completely

consumed the test solution within 2 h after administration [4]. No crystallization of sugar solution occurred [3,4].

Foragers have a lower sucrose requirement when incubated in cages, leading to decreased sucrose consumption in cages as compared to the field. Thus, to test field-relevant CLO and TMX doses approaching a realistic worst-case scenario, we fed foragers with pesticide solutions that were more concentrated (CLO lower: 16 ppb; CLO higher: 80 ppb; TMX lower: 20 ppb; TMX higher: 100 ppb) than those typically found in field nectar. However, we focused on the field-realistic acute doses of CLO and TMX actually ingested by our bees, as recommended by the most recent international guidelines for testing acute oral pesticide exposure [4].

Each test sugar solution contained a different sucrose *quality* (0, 15, 32.5 or 50% w/w) and pesticide treatment (control, lower, or higher dose of CLO or TMX). After pesticide administration, bees fed *limited* quantity diets did not receive any further nutrition. *Ad libitum* diet treatments consisted of bees provided with an *ad libitum* sugar solution in which the concentration was related to the diet quality treatment (15, 32.5 or 50% w/w sucrose concentration).

Survival

We assessed the survival of the bees each minute for the first 10 hours after the administration of the pesticide treatment. Afterwards, we assessed the survival at 24, 48, 72 and 96 hours after treatment. A bee was considered dead when it was immobile and did not react to any stimulation [6]. In total, we tested the survival of 2,840 foragers from five different colonies.

Sugar consumption

Each day, we weighed the sugar syringes. Separately, we used 10 cages maintained in identical conditions, but without bees, to measure the average mass loss due to evaporation of sugar solutions from the syringes. We accounted for this evaporative mass loss (<1%) in our calculations. We calculated the mean daily sugar consumption (g of pure sucrose) per bee. This daily sugar consumption was based on the weight of sugar solution consumed by each cage daily, corrected by the number of alive bees per cage, sugar solution concentration (15%, 32.5% or 50% w/w of sucrose) and sugar solution density (15% = 1059.16, 32.5% = 1139.08, 50% = 1229.65 kg/m^3 [7]). In total, we tested the sugar consumption of 108 groups (cages) of 15 bees (only *ad libitum* treatment).

Glucose and trehalose hemolymph levels

Two hours after the treatments were administered, we captured six live bees per treatment. We exposed each bee to a 40-60% air-CO_2 mixture for few seconds before handling (see above).

We extracted 1 µL of hemolymph per bee by puncturing the intersegmental membrane between the 4th and the 5th abdominal tergite (taking care to not puncture the crop) with a graduated 5 µL microcapillary tube (Blaubrand®, 125 mm length, accuracy ± 0.30 %, reproducibility ± 0.6 %). We used the microcapillary to gently puncture the intersegmental membrane. Subsequently, we collected the hemolymph that freely flowed from the membrane inside the microcapillary [8]. Therefore, we did not directly insert the microcapillary in the abdomen, allowing the specific sampling of hemolymph only: all samples were clear and slightly yellow. The hemolymph was immediately transferred to a 0.5 mL microtube and flash frozen in liquid nitrogen. The samples were then stored at – 80 °C until testing. The *limited* diet treated bees did not provide sufficient hemolymph, perhaps because of dehydration, and we therefore only extracted and analyzed hemolymph from the *ad libitum* diet treatments.

We measured the titer of glucose (linear dynamic range: 0.5-100 µg/µL) and trehalose (0.4-94 µg/µL) [9]. We used a glucose assay kit (Sigma-Aldrich, catalog no. GAHK-20) which contained the enzyme mix (1.5 mM NAD+, 1.0 mM ATP, 1.0 unit/mL of hexokinase, 1.0 unit/mL of glucose-6-phosphate dehydrogenase) and a glucose standard (1 mg/mL). Glucose was phosphorylated by adenosine triphosphate (ATP) to form glucose- 6-phosphate (G6P). G6P was then converted to 6-phosphogluconate in the presence of NAD+, which resulted in an equimolar amount of NAD+ being reduced to NADH. This reduction was detected spectrophotometrically as an increase in absorbance at 340 nm that was directly proportional to the glucose concentration in the sample [10].

Each molecule of trehalose (a disaccharide) was converted to two molecules of D-glucose by the enzyme trehalase (Sigma-Aldrich, USA catalog no. T8778). We added 2 µL of 1:4 diluted trehalase enzyme to each microplate well, which were then wrapped in Parafilm. The microplate was shaken for 60 s (oscillation amplitude of 1 mm) and incubated at 37°C for 21 hours.

We then repeated the glucose assay described above. Glucose standards were prepared before each run by adding 0 (blank), 0.5, 1, 5, 10, 30, 50 and 100 µL of glucose standard solution (1 mg/mL) to seven 1.5 mL microtubes, while the samples were thawed on ice. An appropriate volume of enzyme mix was added to each tube to obtain a total volume of 1000 µL. The tubes were inverted five times and then centrifuged at 18000 RCF (relative centrifugal force) for 30 s to spin down their contents. The samples were maintained at room temperature for 15 minutes. Subsequently, 200 µL of the standards and samples were dispensed in triplicate into a microplate

(Greiner 96 Flat Bottom Transparent Polystyrol, Greiner, Germany) and read with a microplate reader (Infinite 200Pro, Tecan, USA) at 340 nm.

We corrected the absorbance values by subtracting the blank from each absorbance value and we used the arithmetic mean of the absorbance of the three replicates. We generated calibration curves with the known glucose standards and used the following linear regression equations to interpolate the glucose and trehalose concentrations of the unknown samples:

$$[Glucose] = \frac{Abs_{sample} - intercept}{Slope}$$

$$[Trehalose] = \left(\frac{Abs_{sample} - intercept}{Slope} - [Glucose]\right) * \frac{342.3}{(180.2 * 2)}$$

Finally, each trehalose and glucose titer was multiplied by 1000 to account for the initial dilution with the enzyme mix (1 µL of hemolymph plus 999 µL of mix). In total, we measured the trehalose and glucose titers of 216 foragers.

Statistical methods

We used Kaplan-Meier survival analyses (Wilcoxon Chi-square values) to determine the effects of diet quality (*rich*, *intermediate*, *poor*, or *no nutrients*) on the survival of pesticide-free bees exposed to diets of different quantity (*ad libitum* or *limited*). We applied the Dunn-Sidak correction [11] to correct for multiple comparisons ($k = 3$, adjusted $\alpha = 0.0170$, ESM figure S3A, B). We used Kaplan-Meier survival analyses (Wilcoxon Chi-square values) to test the effects of dose of TMX and CLO on the survival of honey bees exposed to diets of different quantity and quality. We applied the Dunn-Sidak correction [11] to correct for multiple comparisons (*ad libitum* trials: $k = 3$, adjusted $\alpha = 0.0170$; *limited* quantity trial: $k = 4$, adjusted $\alpha = 0.0127$, ESM table S1, figure 1). We compared the survival of the bees fed the *no nutrients* diet with only bees fed the *limited* diet (ESM table S1) to reduce the number of comparisons tested, given the expected extreme survival difference between the *ad libitum* and *no nutrients* treatments. In our survival analyses, we censored all bees that were removed (2 h after treatment) for hemolymph sampling.

We used a binomial proportion model [12] to test for synergistic effects of nutritional stress (treatment A) and neonicotinoid exposure (treatment B) on bee survival (figure 2). We used the additive effects model [13], in which synergism is defined as the combined effect of multiple stressors significantly exceeding the sum of effects elicited by individual stressors. The R script (p.adjust function) used is available in the following chapter of our ESM Methods, and further details on this test are described in Sgolastra et al. [12]. We tested for a synergistic effect by testing if the difference between the observed and the expected mortality of the combined treatment (AB)

could arise by chance alone (non-significantly different from zero, null hypothesis) or was larger than the simple additive effect of both stressors (significantly larger than zero, alternative hypothesis).

We used the 0 ng/bee dose treatment as the control reference for the pesticide stress, and the *ad libitum rich* diet treatment as the control reference for the nutritional stress. Treatment A consisted of pesticide-free (*control* dose) bees exposed only to nutritional stress (*ad libitum intermediate*, *ad libitum poor*, *limited intermediate* and *limited poor*). Treatment B consisted of only bees exposed to pesticide stress (higher doses), which were fed the optimal diet treatment (*ad libitum rich*). Bees exposed to both nutritional (*ad libitum intermediate*, *ad libitum poor*, *limited intermediate* and *limited poor)* and pesticide (higher doses) stressors were assigned to the combined treatment (AB). We calculated the expected mortality proportion of the combined treatment as $P_{ABExp} = P_A + (1-P_A) P_B$, where P_A and P_B are the observed mortality proportions in the nutritional and pesticide treatments, respectively. We used Wald confidence intervals to build a hypothesis test for the difference between two proportions. We separately determined the synergistic effects at each assessment time based upon visual data inspection and the Holm method to correct for multiple comparisons ($\alpha = 0.05$). We calculated the Synergistic Effect Sizes (SES) as the difference between observed and expected mortality ratios (ESM table S3).

We used Generalized Linear Models (GLMs) to test the fixed effect of diet quality and colony on sugar consumption (weight, Poisson distribution, reciprocal link, ESM figure S3C), sugar solution consumption (volume, Poisson distribution, reciprocal link, ESM figure S3D), and glucose and trehalose hemolymph levels (Exponential distribution, identity link, ESM figure S3E, F) of pesticide-free bees fed *ad libitum* diets. Separately, for each neonicotinoid (CLO or TMX), we used GLMs to test the fixed effects of pesticide dose and colony on daily sugar consumption (Poisson distribution, reciprocal link, ESM table S4, ESM figure S1) and glucose and trehalose hemolymph levels (Exponential distribution, identity link, ESM table S5, ESM figure S2) of foragers fed *ad libitum* diets of different qualities. We confirmed the suitability of GLM distributions and links with the Pearson goodness-of-fit test and residual analyses. We corrected the model for overdispersion when appropriate [14]. Based upon visual data inspection, effects were further analysed with post-hoc Least-Square Means contrast tests. We used the Dunn-Sidak method to correct for multiple comparisons ($k = 2$, adjusted $\alpha = 0.0253$; $k = 3$, adjusted $\alpha = 0.0170$).

We used R v3.3.2 [15] and JMP v10.0 statistical software, and report mean ± 1 standard error (SE). We indicate with ^{DS} the statistical tests that were corrected using the Dunn-Sidak method.

R script

```
################################################################################
# Testing for additivity:
#     Confidence interval for binomial proportion difference under Bliss independence.
#
# INPUTS:
# ndead = vector with 3 elements, containing number of dead individuals under
#         treatment A, B and combined.
# ntot  = vector with 3 elements, containing total number of individuals under
#         the 3 treatments.
# p.signif = significance level (usually 0.05).
# alternative = character string specifying the alternative hypothesis.
#
# OUTPUTS:
# See Tosi et al.
################################################################################

ci.bliss.additivity <- function(ndead,ntot,p.signif=0.05,alternative="greater") {
  if (alternative=="two.sided") p.signif <- p.signif/2  # Two-tailed test.
  ndead <- unname(ndead)
  ntot <- unname(ntot)
  p <- ndead/ntot
  pa <- p[1]
  pb <- p[2]
  pab.obs <- p[3]
  vara <- p[1]*(1-p[1])/ntot[1]
  varb <- p[2]*(1-p[2])/ntot[2]
  varab.obs <- p[3]*(1-p[3])/ntot[3]
  pab.exp <- pa+pb-pa*pb
  varab.exp <- vara+varb+pb^2*vara+pa^2*varb    # Derived with the Delta method.
  p.dif <- pab.obs-pab.exp
  sd.all <- sqrt(varab.obs+varab.exp)
  z <- qnorm(1-p.signif)
  out <- list(pA=pa,pB=pb,pAB.obs=pab.obs,pAB.exp=pab.exp,p.Dif=p.dif,
```

```
VarA=vara,VarB=varb,VarAB.obs=varab.obs,VarAB.exp=varab.exp,Var.All=sd.all^2,
    CI=switch(alternative,
        two.sided=c(lower=p.dif-z*sd.all,upper=p.dif+z*sd.all),
        less=c(upper=p.dif+z*sd.all),
        greater=c(lower=p.dif-z*sd.all)))
  return(out)
}

# Calculates the exact p-value by inverting the hypothesis test.
invert.hypothesis.bliss <- function(n.mort,n.total) {
  fbliss <- function(signif) ci.bliss.additivity(n.mort,n.total,signif,alternative="greater")$CI["lower"]
  loglik <- function(signif) abs(fbliss(signif))
  return(optimize(loglik,interval=c(0,1),maximum=F,tol=1e-32)$minimum)
}

# Testing ad libitum diet quantity, range of time assessments: 2-24h
# Mortality data. Column 1 (e.g. datamort[[1]][,1]) contains the total number of individuals,
labelled "N".
datamort <- list()
datamort[[1]] <-
cbind(c(90,90,90),c(1,0,17),c(1,1,27),c(1,1,29),c(2,1,32),c(3,1,34),c(5,1,36),c(16,1,45),c(20,1,47),c(
24,1,50),c(34,3,58)) # Nutritional Stress: Ad libitum, 15%; Pesticide: CLO
datamort[[2]] <-
cbind(c(90,90,90),c(0,0,1),c(0,1,9),c(0,1,10),c(0,1,10),c(0,1,11),c(0,1,11),c(0,1,11),c(0,1,12),c(0,1,1
2),c(6,3,28)) # Nutritional Stress: Ad libitum, 32.5%; Pesticide: CLO
datamort[[3]] <-
cbind(c(91,90,91),c(0,0,5),c(0,0,20),c(0,0,25),c(0,0,26),c(0,0,27),c(2,0,27),c(4,1,34),c(9,1,34),c(9,1,
36),c(23,1,40)) # Nutritional Stress: Ad libitum, 15%; Pesticide: TMX
datamort[[4]] <-
cbind(c(90,90,90),c(1,0,0),c(1,0,2),c(1,0,2),c(1,0,2),c(1,0,3),c(1,0,3),c(2,1,3),c(2,1,4),c(2,1,4),c(7,1,
9)) # Nutritional Stress: Ad libitum, 32.5%; Pesticide: TMX
for (i in 1:4) rownames(datamort[[i]]) <- c("TREAT.A","TREAT.B","TREAT.AB") # TREAT.A =
Nutritional stress; TREAT.B = Pesticide stress; TREAT.AB = Combination
```

```
for (i in 1:4) colnames(datamort[[i]]) <-
c("N","2h","3h","4h","5h","6h","7h","8h","9h","10h","24h")

cat("----------------------------------------------------------------\n")

# Testing Bliss additivity. All we need to do is to define "n.total" and "n.mort", and then feed
invert.hypothesis.bliss() with those two numbers.
# Index i runs from 1 to the number of treatments tested (=4).
# For a generic dataset with 1 endpoint and where nt=total number of individuals and nd=number of
dead individuals, we would do: p <- invert.hypothesis.bliss(nt,nd)

for (i in 1:4) {
  a <- datamort[[i]]
  b <- a[,-1]
  p.value <- NULL

# For each endpoint j we test the Bliss hypothesis.
  for (j in 1:10) {
    n.total <- a[c(1,2,3),1]    # Total number of individuals
    n.mort <- a[c(1,2,3),j+1]   # Number of dead individuals.
    p <- invert.hypothesis.bliss(n.mort,n.total)  # p-value from inverting the hypothesis test.
    p.value <- c(p.value,p)
  }
# Control for multiple comparison, Holm methodology. For cases where there is only 1 endpoint
this is obviously not needed.
  p.correct <- p.adjust(p.value,method="holm")

# Formatted output.
  name.data <- c("Nutritional Stress: Ad libitum, 15%; Pesticide: CLO","Nutritional Stress: Ad
libitum, 32.5%; Pesticide: CLO","Nutritional Stress: Ad libitum, 15%; Pesticide:
TMX","Nutritional Stress: Ad libitum, 32.5%; Pesticide: TMX")
  cat(paste(name.data[i],"\n",sep=""))
  names(p.correct) <- c("2h","3h","4h","5h","6h","7h","8h","9h","10h","24h")
  print(datamort[[i]])
```

```
  cat("\n")
  cat(paste(name.data[i],". Observed and expected binomial proportions.\n",sep=""))
  pab <- a[,-1]/a[,1]
  pab <- rbind(pab,pab[1,]+pab[2,]-pab[1,]*pab[2,])
  rownames(pab) <- c("TREAT.A","TREAT.B","TREAT.AB","Expected")
  print(pab)
  cat("\n")
  cat(paste(name.data[i],". Control of type I errors (Holm method) in binomial proportion
test.\n",sep=""))
  print(p.correct)
  cat("-----------------------------------------------------------------\n")
}
#
# Testing limited diet quantity, range of time assessments: 2-10h
# Mortality data. Column 1 (e.g. datamort[[1]][,1]) contains the total number of individuals,
labelled "N".
datamort <- list()
datamort[[1]] <-
cbind(c(91,90,90),c(2,0,11),c(8,1,41),c(31,1,57),c(50,1,67),c(69,1,81),c(77,1,87),c(81,1,88),c(84,1,
90),c(84,1,90)) # Nutritional Stress: Limited, 15%; Pesticide: CLO
datamort[[2]] <-
cbind(c(90,90,90),c(0,0,0),c(0,1,8),c(0,1,36),c(20,1,66),c(63,1,77),c(74,1,80),c(82,1,83),c(86,1,86),
c(87,1,86)) # Nutritional Stress: Limited, 50%; Pesticide: CLO
datamort[[3]] <-
cbind(c(90,90,90),c(2,0,7),c(9,0,51),c(18,0,61),c(36,0,67),c(57,0,77),c(69,0,84),c(76,1,86),c(79,1,8
6),c(82,1,86)) # Nutritional Stress: Limited, 15%; Pesticide: TMX
datamort[[4]] <-
cbind(c(90,90,90),c(0,0,2),c(1,0,10),c(20,0,36),c(41,0,64),c(56,0,78),c(70,0,80),c(76,1,82),c(78,1,8
2),c(81,1,85)) # Nutritional Stress: Limited, 50%; Pesticide: TMX
for (i in 1:4) rownames(datamort[[i]]) <- c("TREAT.A","TREAT.B","TREAT.AB") # TREAT.A =
Nutritional stress; TREAT.B = Pesticide stress; TREAT.AB = Combination
for (i in 1:4) colnames(datamort[[i]]) <- c("N","2h","3h","4h","5h","6h","7h","8h","9h","10h")

cat("-----------------------------------------------------------------\n")
```

```
# Testing Bliss additivity (see above).
# Index i runs from 1 to the number of treatments tested (=4).

for (i in 1:4) {
  a <- datamort[[i]]
  b <- a[,-1]
  p.value <- NULL

  for (j in 1:9) {
    n.total <- a[c(1,2,3),1]    # Total number of individuals
    n.mort <- a[c(1,2,3),j+1]   # Number of dead individuals.
    p <- invert.hypothesis.bliss(n.mort,n.total)  # p-value from inverting the hypothesis test.
    p.value <- c(p.value,p)
  }
# Control for multiple comparison, Holm methodology (see above).
  p.correct <- p.adjust(p.value,method="holm")

# Formatted output.
  name.data <- c("Nutritional Stress: Limited, 15%; Pesticide: CLO","Nutritional Stress: Limited, 
50%; Pesticide: CLO","Nutritional Stress: Limited, 15%; Pesticide: TMX","Nutritional Stress: 
Limited, 50%; Pesticide: TMX")
  cat(paste(name.data[i],"\n",sep=""))
  names(p.correct) <- c("2h","3h","4h","5h","6h","7h","8h","9h","10h")
  print(datamort[[i]])
  cat("\n")
  cat(paste(name.data[i],". Observed and expected binomial proportions.\n",sep=""))
  pab <- a[,-1]/a[,1]
  pab <- rbind(pab,pab[1,]+pab[2,]-pab[1,]*pab[2,])
  rownames(pab) <- c("TREAT.A","TREAT.B","TREAT.AB","Expected")
  print(pab)
  cat("\n")
  cat(paste(name.data[i],". Control of type I errors (Holm method) in binomial proportion 
test.\n",sep=""))
```

```
  print(p.correct)
  cat("-----------------------------------------------------------------\n")
}
```

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ESM TABLES

ESM table S1. Survival of bees exposed to sublethal field-realistic neonicotinoid doses and fed sugar diets of different quantity and quality. We report the Lethal Time at which 25%, 50%, and 75% (LT_{25}, LT_{50}, and LT_{75}) of bees died for each treatment, as well as their short term (1, 2 and 3 h after treatment) mortality as percentages. We tested the *no nutrients* diet (0% sucrose concentration) to include a scenario in which bees had no nutrients available. We state Not Applicable (NA[1]) when the respective LT was not reached because of bee mortality, or (NA[2]) to indicate that the comparisons between the *no nutrients* and the *ad libitum* diets were not tested, given the extreme survival difference between treatments. Different letters next to the lethal times indicate significant differences of each respective treatment (Kaplan-Meier[DS] test).

Diet quantity	Neonicotinoid	Diet quality (%)	N	DF	χ^2	*P*-value	LT_{25} - LT_{50} - LT_{75} (h)			
							Control dose	Lower dose	Higher dose	No nutrients
Ad libitum	**TMX**	**Rich**	270	2	1.3	0.5164	96-NA[1]-NA[1]	96-NA[1]-NA[1]	96-NA[1]-NA[1]	NA[2]
		Intermediate	270	2	0.1	0.9904	48-72-96	48-72-NA[1]	48-72-96	
		Poor	272	2	19.0	0.0003	24-48-72 a	24-48-72 a	4-48-72 b	
	CLO	**Rich**	270	2	4.2	0.1250	96-NA[1]-NA[1]	72-NA[1]-NA[1]	72-96-NA[1]	NA[2]
		Intermediate	270	2	14.4	0.0025	48-72-NA[1] a	48-48-NA[1] ab	24-48-NA[1] b	
		Poor	270	2	42.4	<0.0001	8-48-96 a	8-36-48 a	3-8-48 b	
Limited	**TMX**	**Rich**	405	2	31.4	<0.0001	4-5-7 a	4-5-6 a	4-4-5 b	4-4-5 b
		Poor	407	2	68.3	<0.0001	4-5-6 a	4-5-7 a	2-3-5 b	4-4-5 c
	CLO	**Rich**	405	2	58.2	<0.0001	5-5-6 a	4-5-6 a	4-4-5 b	4-4-5 b
		Poor	406	2	46.2	<0.0001	4-5-6 a	4-5-6 a	2-3-5 b	4-4-5 a

ESM table S2. Effects of sublethal field-realistic neonicotinoid doses and sugar diets of different quantity and quality on bee mortality at 1 h, 2 h and 3 h after treatment.

<table>
<tr><th rowspan="3">Diet quantity</th><th rowspan="3">Neonicotinoid</th><th rowspan="3">Diet quality (%)</th><th rowspan="3">N</th><th colspan="12">Mortality (%)</th></tr>
<tr><th colspan="3">Control</th><th colspan="3">Lower</th><th colspan="3">Higher</th><th colspan="3">No nutrients</th></tr>
<tr><th>1 h</th><th>2 h</th><th>3 h</th><th>1 h</th><th>2 h</th><th>3 h</th><th>1 h</th><th>2 h</th><th>3 h</th><th>1 h</th><th>2 h</th><th>3 h</th></tr>
<tr><td rowspan="6">Ad libitum</td><td rowspan="3">TMX</td><td>Rich</td><td>270</td><td>1</td><td>1</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td rowspan="10">1</td><td rowspan="10">6</td><td rowspan="10">16</td></tr>
<tr><td>Intermediate</td><td>270</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td></tr>
<tr><td>Poor</td><td>272</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>0</td><td>2</td><td>7</td></tr>
<tr><td rowspan="3">CLO</td><td>Rich</td><td>270</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Intermediate</td><td>270</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>3</td></tr>
<tr><td>Poor</td><td>270</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>6</td><td>10</td></tr>
<tr><td rowspan="4">Limited</td><td rowspan="2">TMX</td><td>Rich</td><td>405</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>4</td></tr>
<tr><td>Poor</td><td>407</td><td>0</td><td>1</td><td>3</td><td>0</td><td>1</td><td>4</td><td>0</td><td>3</td><td>19</td></tr>
<tr><td rowspan="2">CLO</td><td>Rich</td><td>405</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>3</td></tr>
<tr><td>Poor</td><td>406</td><td>1</td><td>1</td><td>3</td><td>0</td><td>0</td><td>3</td><td>0</td><td>4</td><td>15</td></tr>
</table>

ESM table S3. Synergistic Effect Sizes (SES) of combined nutritional and pesticide stressors, in relation to time from exposure (1-72 h). For each time assessment, the synergistic effect size was calculated as the difference between observed and expected mortality.

Nutritional Stress	Pesticide	Synergistic Effect Sizes at different times (h) after exposure (%)												
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**24**	**48**	**72**
Ad libitum	CLO	1	1	9	10	10	11	11	11	12	12	21	7	0
intermediate	TMX	0	-1	1	1	1	2	2	0	1	1	1	-11	-16
Ad libitum	CLO	1	18	28	30	32	33	33	31	29	28	25	14	7
poor	TMX	0	5	22	27	29	30	27	32	26	29	18	11	4
Limited	CLO	0	0	8	39	50	15	6	1	0	-1	0	0	0
rich	TMX	0	2	10	18	26	24	11	6	4	4	0	0	0
Limited	CLO	-2	10	36	29	19	14	12	9	8	8	2	1	1
poor	TMX	-1	6	47	48	34	22	17	11	8	4	2	1	0

ESM table S4. Main effects of sublethal field-realistic neonicotinoid dose on average daily sucrose consumption of foragers fed different diet qualities. The asterisk indicates a significant effect of dose (GLMs).

Neonicotinoid	Diet quality	N	DF numerator	DF denominator	L-R χ^2	*P*-value
CLO	Rich	18	5	2	0.24	0.8875
	Intermediate	18	5	2	3.95	0.1391
	Poor	16	5	2	63.52	<0.0001*
TMX	Rich	18	5	2	1.92	0.3820
	Intermediate	18	5	2	2.01	0.3667
	Poor	17	5	2	1.47	0.4805

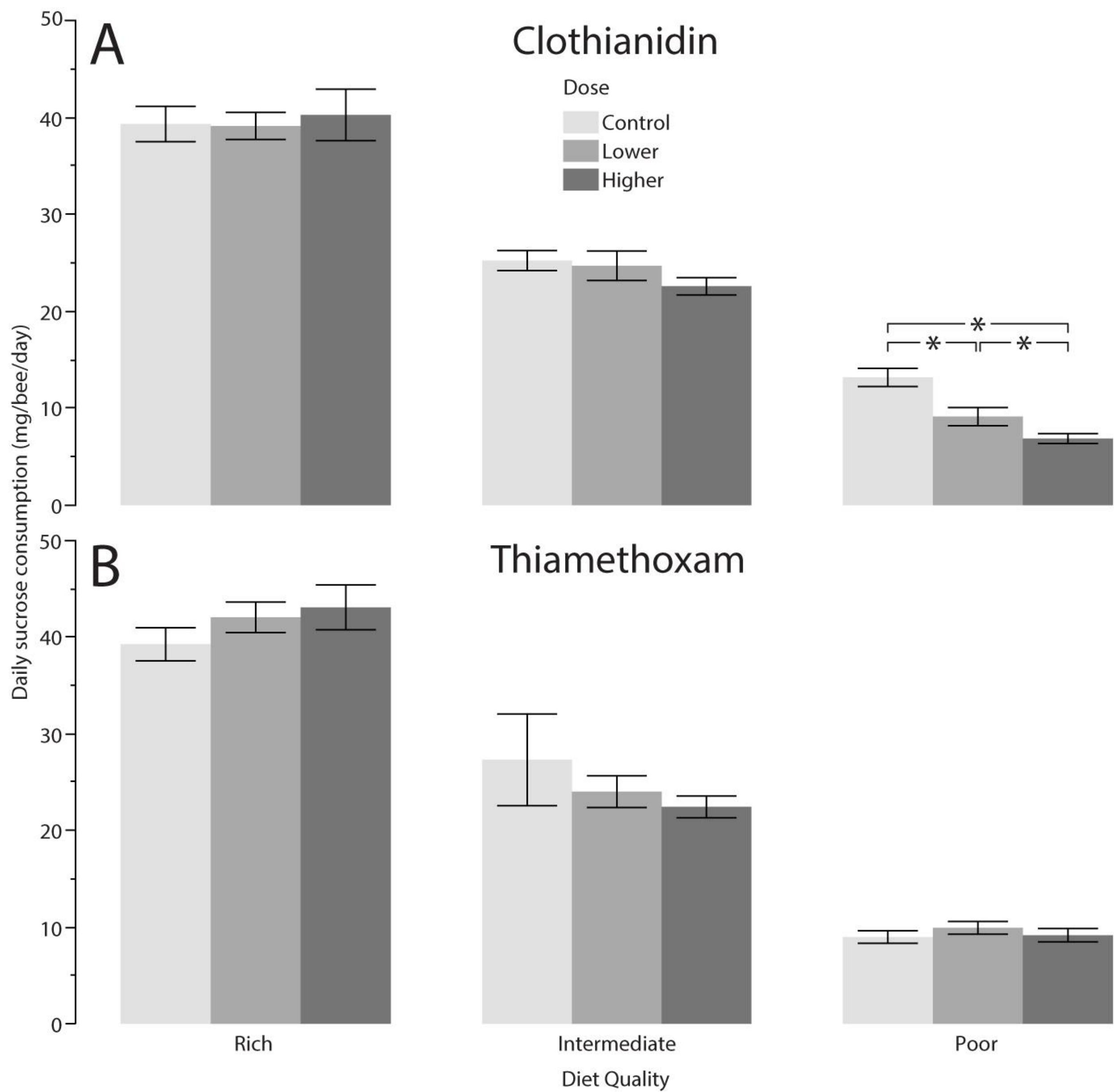
ESM table S5. Main effects of sublethal field-realistic dose of two neonicotinoids (CLO and TMX) on glucose and trehalose levels in forager hemolymph. Results are shown for each diet quality. Asterisks indicate significant effects of dose (GLMs).

Neonicotinoid	Carbohydrate	Diet quality	N	DF numerator	DF denominator	L-R χ^2	*P*-value
TMX	Glucose	Rich	36	5	2	8.82	0.0122*
		Intermediate	36	5	2	0.22	0.8945
		Poor	36	5	2	0.49	0.7822
	Trehalose	Rich	36	5	2	1.16	0.5598
		Intermediate	36	5	2	0.40	0.8194
		Poor	36	5	2	2.53	0.2827
CLO	Glucose	Rich	36	5	2	9.38	0.0092*
		Intermediate	36	5	2	0.89	0.6392
		Poor	36	5	2	2.74	0.2535
	Trehalose	Rich	36	6	2	12.35	0.0021*
		Intermediate	36	6	2	2.33	0.3124
		Poor	36	6	2	5.92	0.0517

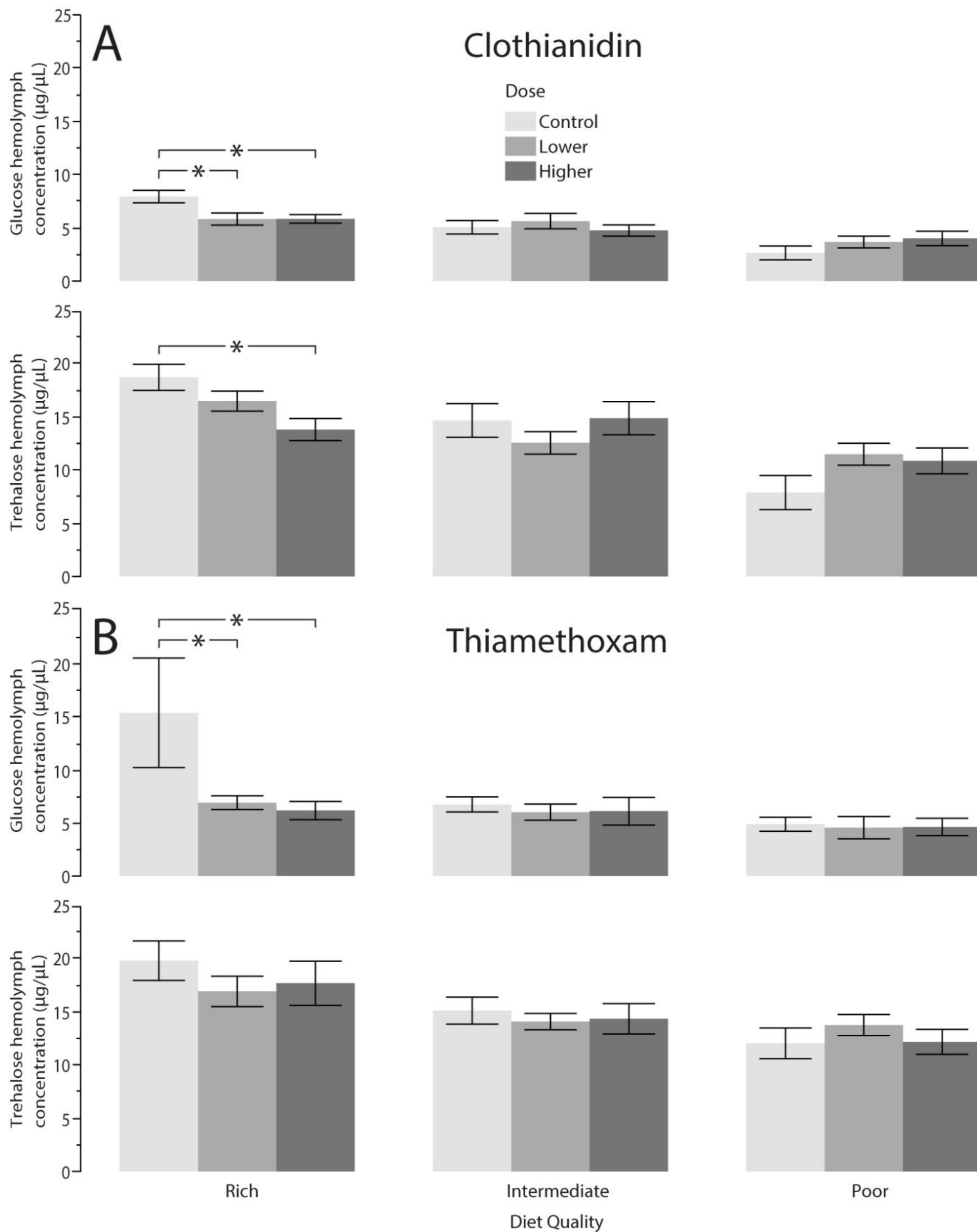
ESM table S6. Effects of diet quantity and quality on the survival of pesticide-free bees. We report the Lethal Time (LT) at which 25%, 50%, and 75% (LT25, LT50, and LT75) of bees died for each treatment. We tested the *no nutrients* diet (0% sucrose concentration) to include a scenario in which bees had no sugar available.

Diet quantity	N	DF	χ^2	*P*-value	LT25 - LT50 - LT75 (h)			
					Rich	Intermediate	Poor	No nutrients
Ad libitum	541	2	119.5	< 0.0001	96-NA-NA a	48-72-NA b	24-48-72 c	
Limited	496	2	33.5	< 0.0001	5-5-6 a		4-5-6 b	4-4-5 c

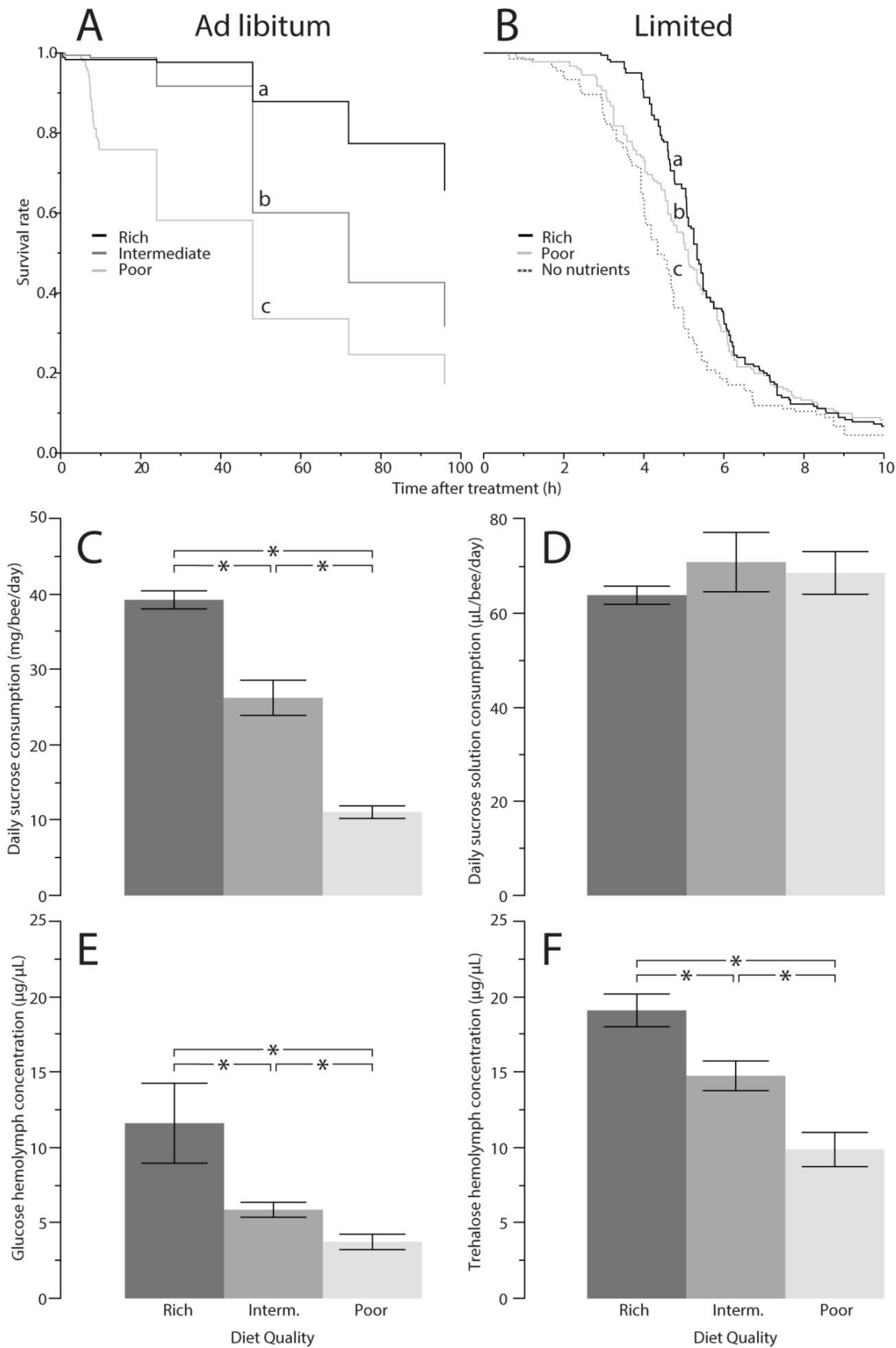
ESM FIGURES



ESM figure S1. Daily sucrose consumption of bees exposed to combined nutritional and pesticide stressors. We exposed bees to three sublethal doses of either CLO (A) or TMX (B), and then we fed them *ad libitum* quantity diets of three different qualities for four days. Darker shading reflects higher doses of pesticide. Asterisks indicate significant differences (GLM, Least-Square Means contrast^{DS} tests). Main effects and sample sizes are shown in ESM table S4. Error bars show standard errors.



ESM figure S2. Glucose and trehalose concentrations in the hemolymph of bees exposed to a combination of nutritional and pesticide stressors. We exposed bees to three sublethal doses of either CLO (A) or TMX (B), and then we fed them *ad libitum* quantity diets of three qualities. The hemolymph was sampled 2 h after the pesticide treatment. Darker shading reflects higher doses of pesticide. Asterisks indicate significant differences (GLM, Least-Square Means contrast[DS] tests). Main effects and sample sizes are shown in ESM table S5. Error bars show standard errors.



ESM figure S3. Effect of diet on (A, B) survival, (C, D) food consumption and (E, F) sugar hemolymph levels of pesticide-free bees. We tested the effects of *rich* (50% sucrose solution),

intermediate (32.5%), *poor* (15%) or *no nutrients* (0%, only limited survival trial, dotted line) quality diets. Darker shading reflects the increased sugar concentration in the diets. We show the (A, B) survival of pesticide-free bees fed (A) *ad libitum* and (B) *limited* quantity diets. Because of the low survival rate and to facilitate graphical display, the survival of bees fed (B) *limited* quantity diets is shown until 10 h after treatment only. In (A, B), different letters indicate significant differences (Kaplan-Meier[DS]; $N_{Limited, Rich} = 180$, $N_{Limited, Poor} = 181$, $N_{Limited, no nutrients} = 135$, $N_{Ad libitum, Rich} = 180$, $N_{Ad libitum, Intermediate} = 180$, $N_{Ad libitum, Poor} = 181$). We measured the daily (C) mass of sucrose consumed and (D) volume of sucrose solution consumed by bees fed *ad libitum* quantity diets of different quality during their 4-day incubation. We sampled the (E, F) hemolymph of bees fed *ad libitum* quantity diets of different quality 2 h after the pesticide treatment. In (C, D, E, F), darker bar shading reflects higher diet sucrose concentration, asterisks indicate significant differences, and error bars show standard errors (GLM, Least-Square Means contrast tests[DS]; (C, D) $N_{Rich} = 12$, $N_{Intermediate} = 12$, $N_{Poor} = 12$; (E, F) $N_{Rich} = 72$, $N_{Intermediate} = 72$, $N_{Poor} = 72$).