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Cite this article: Tosi S, Nieh JC, Sgolastra F, Cabbri R, Medrzycki P. 2017 Neonicotinoid pesticides and nutritional stress synergistically reduce survival in honey bees. *Proc. R. Soc. B* **284**: 20171711.
http://dx.doi.org/10.1098/rspb.2017.1711

Received: 28 July 2017
Accepted: 28 November 2017

Subject Category:
Ecology

Subject Areas:
ecology, environmental science, physiology

Keywords:
bee health, carbohydrates, clothianidin, thiamethoxam, haemolymph, food consumption

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Electronic supplementary material is available online at https://dx.doi.org/10.6084/m9.figshare.c.3951877.

THE ROYAL SOCIETY PUBLISHING

Neonicotinoid pesticides and nutritional stress synergistically reduce survival in honey bees

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The honey bee is a major pollinator whose health is of global concern. Declines in bee health are related to multiple factors, including resource quality and pesticide contamination. Intensive agricultural areas with crop monocultures potentially reduce the quality and quantity of available nutrients and expose bee foragers to pesticides. However, there is, to date, no evidence for synergistic effects between pesticides and nutritional stress in animals. The neonicotinoids clothianidin (CLO) and thiamethoxam (TMX) are common systemic pesticides that are used worldwide and found in nectar and pollen. We therefore tested if nutritional stress (limited access to nectar and access to nectar with low-sugar concentrations) and sublethal, field-realistic acute exposures to two neonicotinoids (CLO and TMX at 1/5 and 1/25 of LD_{50}) could alter bee survival, food consumption and haemolymph sugar levels. Bee survival was synergistically reduced by the combination of poor nutrition and pesticide exposure (−50%). Nutritional and pesticide stressors reduced also food consumption (−48%) and haemolymph levels of glucose (−60%) and trehalose (−27%). Our results provide the first demonstration that field-realistic nutritional stress and pesticide exposure can synergistically interact and cause significant harm to animal survival. These findings have implications for current pesticide risk assessment and pollinator protection.

1. Introduction

Pollinators provide essential ecosystem services, contributing to wild plant biodiversity [1] and sustaining agricultural productivity [2]. The honey bee is a major pollinator species, and its poor health is related to multiple factors [3,4], including resource quality [5] and pesticide contamination [6]. Concern is therefore growing about honey bee nutrition and the potential for synergistic effects between pesticide exposure and nutrition [7,8].

Intensive agriculture with crop monocultures modifies natural land use, reduces natural habitats and plant diversity [9], and decreases the quality and quantity of nutrients in nectar and pollen [7,10]. Honey bees pollinate multiple crops and can therefore be vulnerable to such reduced food quality. Nutritional stress plays a crucial role in bee losses and poor colony health [7,11]. In fact, nutritional deficits were identified as a major cause of colony losses in the USA between 2007 and 2015 (21–58%) [12].

Agriculture also exposes foragers to pesticides [13]. Attention has focused on the neonicotinoid pesticides [14] because of their adverse impacts on pollinator health [15]. Neonicotinoids are globally used systemic insecticides [16] that can be found in the nectar and pollen collected by foragers [17], and are

highly toxic to bees [16]. Bees can be exposed to pesticides that drift from treated fields when they forage on flowering strips, buffer zones, and cover or catch crops [18]. Furthermore, neonicotinoids are highly persistent and are found in environmental reservoirs such as water and soil [17]. Consequently, plants could take up neonicotinoids years after the actual treatment, resulting in prolonged contamination [13,17].

Clothianidin (CLO) and thiamethoxam (TMX) are commonly used neonicotinoids, and CLO is also a degradation product of TMX [16]. These neurotoxic insecticides are agonists of nicotinic acetylcholine receptors (nAChRs) [16] and impair bees in multiple ways [15,19]. Neonicotinoids have additive and synergistic effects on honey bees in combination with health stressors such as nosemosis and *Varroa* infestation (for review, see [20]). Moreover, the combination of poor nutrition and pesticide exposure may be especially problematic given that some genes can be upregulated by pesticide or pollen stress [21]. To date, there is no evidence for negative synergistic effects between pesticides and nutritional stressors in any animal studies [22]. However, good nutrition can help: bees were typically more resistant to pesticides when fed pollen diets [21,23]. Food quality can influence the effect of toxins on the health of other arthropods, such as *Daphnia* [24–26] and *Diaptornus* [27]. A few studies have demonstrated synergies between starvation and contamination from heavy metals, PAHs or PCBs on aquatic animals (fish, amphipods and molluscs) (for review, see [22]). However, these are not pesticides. We therefore decided to study the interactive effects of field-realistic neonicotinoids and nutritional stress on a major pollinator species.

We focused on honey bees because they are an important pollinator and are an indicator of how insect pollinators can respond to environmental stressors [28]. Bee foragers are particularly important because they are the only colony members that spend a significant proportion of their time flying [29] and therefore have significant energy needs. Unlike other insects, in which flight is initially powered by glycogen and subsequently by lipids, honey bee flight is entirely powered by sugars in the honey stomach after the depletion of glycogen reserves [30]. Sugar is therefore essential for foraging because flight has high-energetic demands [31,32]: forager metabolic activity increases 50–100 times during flight [31]. A bee could need up to 12 mg of sugar to sustain itself for each 1 h of flight [33]. To deal with such high energy demands, sugars are quickly absorbed into the bee's haemolymph [34].

Honey bees store only small amounts of glycogen in their flight muscles [35] and thus have high haemolymph sugar levels relative to other insects [36]. Haemolymph sugar content is therefore a good indicator of bee nutritional and physiological status. Trehalose, a disaccharide composed of two D-glucose molecules, is the most abundant sugar in honey bee haemolymph [36,37] and can be rapidly metabolized into D-glucose to release energy [37]. D-glucose is another major component of bee haemolymph [38] and is used to power motor activities directly [39].

We therefore tested the combined effects of sublethal, field-realistic acute exposures (see Material and methods) to two neonicotinoids (CLO and TMX at 1/5 and 1/25 of their LD_{50}) and nutritional stress (limited sugar quantity and quality) on forager survival, food consumption and haemolymph sugar levels. Haemolymph sugar levels were assessed 2 h after treatment to test for potential rapid alterations caused by pesticide administration. Survival and sugar consumption were assessed over a longer period (4 days). We studied foragers because they spend a majority of their time foraging, an energy-intensive task [31] that can also expose bees to neonicotinoid-contaminated nectar.

2. Material and methods

This study was conducted in the summer of 2015 in Bologna, Italy. We used five queen-right honey bee (*Apis mellifera ligustica*) colonies located in the experimental apiary of the Council for Agricultural Research and Economics, Agriculture and Environment Research Centre (CREA-AA). The colonies were healthy, produced honey and showed no sign of disease throughout the season. They were managed according to an organic production protocol [40], and we used standard inspection techniques [41] to confirm that our colonies did not have detectable disease or parasite infestations. Colonies were inspected at least once per week.

We exposed bees to a nutritional stress (*limited* access to nectar or *ad libitum* access to nectar with low-sugar concentrations) and a neonicotinoid treatment. These treatments were administered individually and in combination to test for synergistic interactions [42]. After exposure, we measured the effects of the nutritional and neonicotinoid stressors on survival (up to 4 days after treatment), food consumption (up to 4 days after treatment), and glucose and trehalose haemolymph levels (2 h after treatment). We repeated the experiment four times (twice for each pesticide), using a total of 2840 foragers from five different colonies. We report mean $\pm$ 1 s.e., and superscript 'DS' indicates the statistical tests that passed the Dunn–Sidak correction for multiple pairwise comparisons. Further details are reported in the electronic supplementary material.

(a) Sugar diet treatments

We define nutritional stress as limited access to nectar or access to nectar with low-sugar concentrations. We tested sugar diets with different quantities (amounts) and qualities (concentrations) of sucrose. We provided the bees either *ad libitum* or *limited* (10 µl) quantities of sugar solution. The quality of the sugar diet was either *rich* (50% (w/w) sucrose solution), *intermediate* (32.5%) or *poor* (15%).

Our nutritional stresses are field-realistic. Foragers can be exposed to the sugar concentrations that we tested when foraging for nectar or consuming non-ripened honey stored in the nest. Bees collect nectar containing 5–80% (w/v) sugar concentration [43,44], but sugar concentrations can be as low as 2% [43]. Nectar is converted into honey in the hive via ripening, a process that increases sugar concentrations [44]. However, this process starts in the hive only [44]. Counterintuitively, foragers even dilute the sugar concentration in nectar by approximately 1% during nectar collection [45]. Thus, foragers can consume nectar containing less than 5% sugar while foraging and flying outside the nest.

Inside the nest, nectar is ripened gradually over a period taking up to 5 [45] or even 21 days [44]. When nectar is rapidly collected in large quantities, bees do not immediately ripen it; instead they deposit the nectar, largely unconcentrated, into storage cells [45]. Ripening is therefore influenced by multiple factors: weather, honey flow conditions, collection rates, colony strength, amount and concentration of nectar, extent of available storage cells, temperature, humidity and ventilation conditions [45]. Bees can thus be exposed to largely unconcentrated nectar for several days when consuming carbohydrates stored in the hive.

Individual carbohydrate intake can also be limited by non-foraging periods. In fact, lack of sufficient food stores is a common cause of winter colony losses [11] (i.e. involved in 58% of the colonies lost in the USA in 2014–2015 [12]). In our study, we therefore tested this limited carbohydrate scenario in two ways: feeding bees with a limited amount of sucrose solution or, in a separate treatment, feeding bees *no nutrients* (0% sucrose).

(b) Neonicotinoid treatments

We followed the most recent international guidelines for pesticide tests on bees [46]. We tested sublethal acute oral exposure to field-realistic doses of two neonicotinoid pesticides: CLO and TMX. Our doses were field-realistic because bees can consume higher doses of CLO and TMX while collecting contaminated nectar in the field for a short period (1 h) (see details below). Treatments consisted of a control dose (pesticide-free) or a neonicotinoid dose (dose) that was either 1/25 (lower dose, TMX = 0.2 ng/bee, CLO = 0.16 ng/bee) or 1/5 (higher dose, TMX = 1 ng/bee, CLO = 0.8 ng/bee) of their respective LD_{50} (TMX = 5 ng/bee, CLO = 4 ng/bee) [47,48]. The *no nutrients* diet was pesticide-free. The higher doses used for each neonicotinoid reflect field-realistic scenarios with elevated neonicotinoid contamination. Calculations based on European Food Safety Authority (EFSA) [49] data confirm that our sublethal doses were lower than the worst-case scenario in which bees foraged for 1 h on nectar that was contaminated with CLO or TMX after a seed treatment (maximum field-realistic doses: CLO = 1 ng/bee/1 h, TMX = 0.66 ng/bee/1 h) or a transplant-drip application (maximum field-realistic dose of TMX = 1.80 ng/bee/1 h).

For CLO, the EFSA [49] calculated that foragers can consume up to 1 ng/bee in 1 h of nectar foraging. This calculation was based on the field-realistic concentration of CLO in nectar (9 ppb, found in oilseed rape nectar after seed treatment application [49]) and sugar in oilseed rape nectar (10% (w/w) [44,50]). A previous study similarly estimated that a forager can acutely consume up to 1.36 ng of CLO in a foraging trip when collecting nectar on oilseed rape fields grown from seeds treated with CLO [42]. In fact, CLO can occur at even higher field-realistic concentrations in nectar (e.g. 10 ppb [17,51]) and pollen (e.g. 41 ppb [52]) than those used in our study.

Similarly, for TMX, EFSA [49] calculated that foragers can consume up to 0.66 ng/bee in 1 h of foraging for nectar (10% (w/w) sugar, oilseed rape) with 5 ppb of TMX (concentration found in nectar after seed treatment application [49]). However, foragers can consume up to 1.80 ng/bee in 1 h of foraging for nectar with 15 ppb of TMX (concentration found in nectar after transplant-drip application [51]). TMX also is found at higher concentrations in nectar (e.g. 17 ppb [52]; 19 ppb [51]; 20 ppb [53]) and pollen (e.g. 127 ppb [52]) than those used in our study. Further details on our neonicotinoid treatments are provided in the electronic supplementary material.

3. Results

(a) Combined nutritional and neonicotinoid stressors synergistically reduced survival

Survival was monitored up to 4 days after exposure to the neonicotinoids. Sublethal and field-realistic doses of neonicotinoids did not significantly reduce survival when foragers were fed *ad libitum rich* diets (Kaplan–Meier, $p > 0.13$; electronic supplementary material, table S1; figure 1*a*,*f*). However, neonicotinoids significantly reduced the survival of bees fed the *ad libitum* diets with qualities that were *intermediate* (CLO; figure 1*b*) or *poor* (CLO and TMX, Kaplan–Meier, $p < 0.01$; figure 1*c*,*h*). Bees fed *higher* pesticide doses had significantly lower survival when compared with *control* bees (CLO: within *poor*- and *intermediate*-quality diets groups; TMX: within the *poor*-quality diet group) and *lower* dose (CLO: within the *poor*-quality diet group) ($p < 0.0170$, Kaplan–Meier^{DS}).

CLO and TMX also reduced the survival of bees fed *limited*-quantity diets with either *rich* (figure 1*d*,*i*) or *poor* (figure 1*e*,*j*) sugar qualities (Kaplan–Meier, $p < 0.0001$; electronic supplementary material, table S1). Specifically, *higher* doses of both neonicotinoids significantly reduced survival when compared with *control* and *lower* doses, at all diet qualities ($p < 0.0170$, Kaplan–Meier^{DS}). Increased death of bees fed neonicotinoids and *poor*-quality diets occurred 2–3 h after treatment (up to 0%, 6% and 19% mortality, respectively, 1 h, 2 h and 3 h after treatment; electronic supplementary material, table S2).

There was a significant synergistic reduction in survival elicited by all combinations of nutritional stresses (*ad libitum intermediate*, *ad libitum poor*, *limited high* and *limited poor*) and the *higher* pesticide dose (binomial proportion test, Holm correction; figure 2; electronic supplementary material, table S3). *Ad libitum poor* diets synergistically reduced survival between 2–24 h (CLO and TMX, $SES_{range} = 5–33\%$; figure 2*b*,*f*), and *ad libitum intermediate* diets synergistically reduced survival between 3–24 h (CLO, $SES_{range} = 9–21\%$; figure 2*a*). There was no significant synergistic effect on the survival of bees exposed to the *ad libitum intermediate* diet and TMX. *Limited poor* diets synergistically reduced survival between 2–10 h (CLO, $SES_{range} = 8–36\%$; figure 2*d*) and 3–8 h (TMX, $SES_{range} = 11–48\%$; figure 2*h*), and *limited rich* diets synergistically reduced survival between 4–5 h (CLO, $SES_{range} = 39–50\%$; figure 2*c*) and 3–6 h (TMX, $SES_{range} = 10–24\%$; figure 2*g*).

Receiving no nutrients (i.e. starvation) was better than receiving some nutrients with pesticides. Within the *limited*-quantity diet trial, we tested an additional diet containing *no nutrients* (10 µl of pure water). Bees fed the *no nutrients* diet had significantly higher survival than those fed the *limited*-quantity diet of *poor* quality (10 µl of 15% sucrose solution) containing the *higher* pesticide dose of either CLO or TMX (electronic supplementary material, table S1 and figure 1*e*,*j*). The survival of bees fed the *no nutrients* diet was significantly lower than that of bees fed *limited poor* diets containing the *control* and *lower* dose (TMX: at 15–50%; CLO: at 50%; figure 1).

(b) Combined nutritional and neonicotinoid stressors reduced sugar consumption

We assessed the sucrose consumption of bees fed the *ad libitum* diet only because bees that received a *limited*-quantity diet only had access to a fixed amount of food (10 µl). We calculated the actual mass of pure sucrose consumed per bee per day. There was no significant effect of CLO on sugar consumption of foragers fed *rich*- and *intermediate*-quality diets (GLMs, $p > 1.40$; electronic supplementary material, table S4 and figure S1A). However, there was a significant effect of CLO on consumption of bees fed a *poor*-quality diet (GLMs, $p < 0.0001$; electronic supplementary material, figure S1A). Specifically, *control* bees consumed significantly more sucrose than *lower* (−31%) and *higher* (−48%) dose

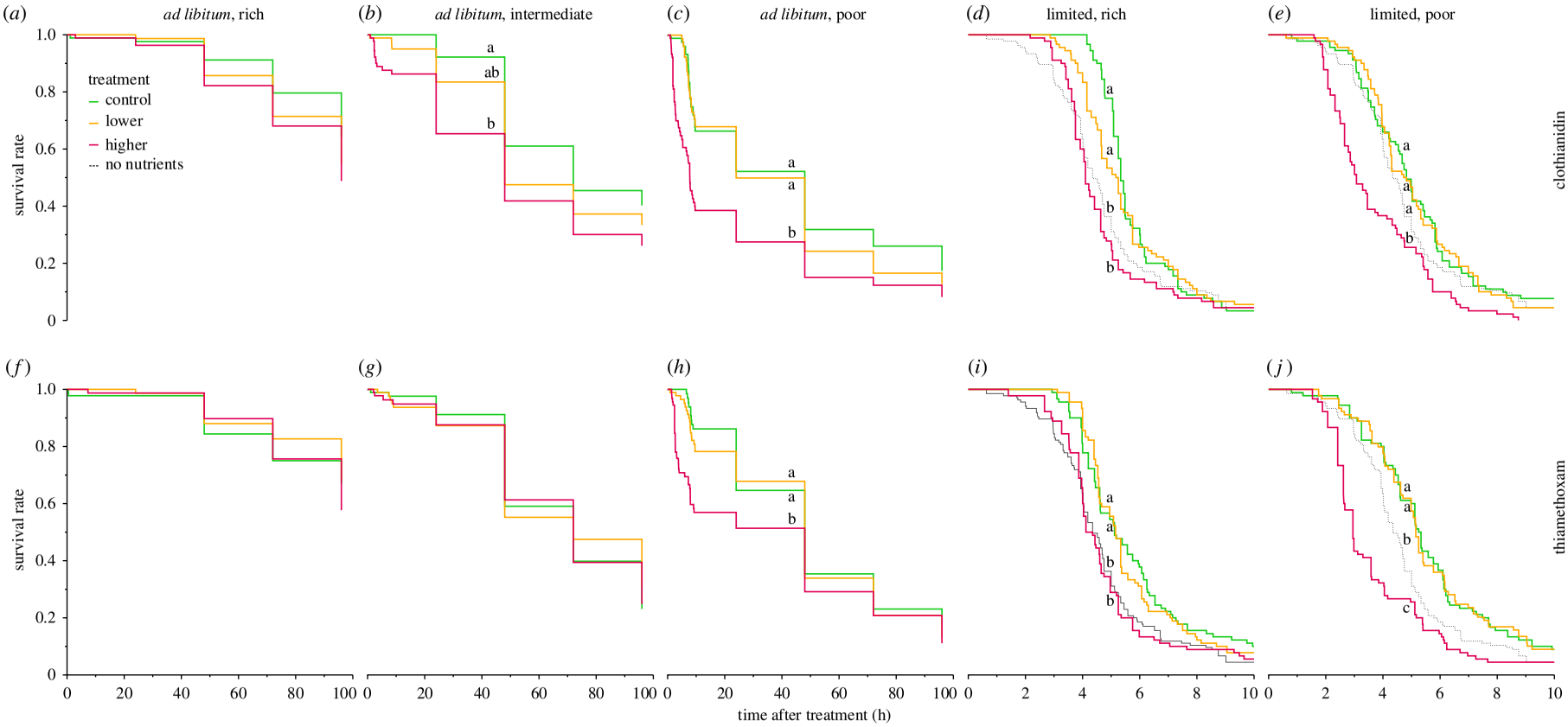

Figure 1. Survival of bees exposed to combined nutritional and pesticide stressors. We exposed bees to a control (green lines), lower (orange) or higher (red) field-realistic sublethal dose of CLO (*a*–*e*) or TMX (*f*– *j*). We fed bees *ad libitum* diets of *rich* (*a*,*f*), *intermediate* (*b*,*g*) or *poor* (*c*,*h*) quality, and *limited* diets of *rich* (*d*,*i*) or *poor* (*e*,*j*) quality. Because of the low survival rate and to facilitate graphical display, we show the survival of bees fed *limited* diets until 10 h after treatment only. We also fed bees a *no nutrients* diet (0% sucrose concentration, dotted dark grey lines), and their survival was compared to bees fed *limited* diets. Different letters indicate significant differences (Kaplan–Meier^{DS} test). Main effects and sample sizes are shown in electronic supplementary material, table S1.

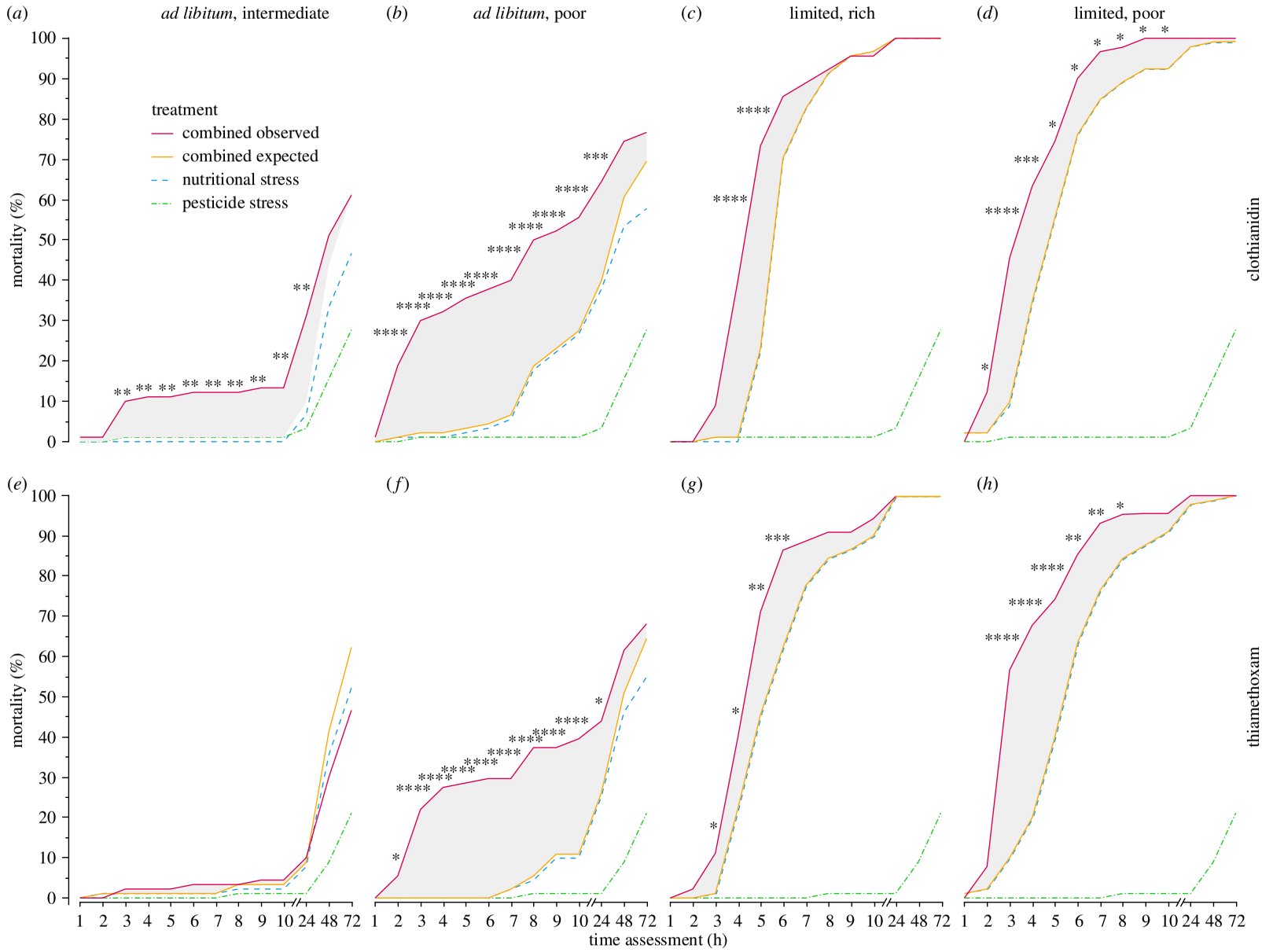

Figure 2. Synergistic effects of nutritional and neonicotinoid stressors on bee survival across time. We tested the individual and combined effects of each nutritional stress (treatment A, blue, dashed lines) and the higher neonicotinoid sublethal field-realistic dose of either CLO (*a–d*) or TMX (*e–h*) (treatment B, green, dashed and dotted lines), and compared their expected (orange, full lines) and observed (red, full lines) combined effects (treatment AB). The size of the synergistic effects is highlighted by the grey-shaded area between expected and observed mortality. Asterisks indicate significant synergistic effects (i.e. significant difference between mortality of expected and observed combined treatment) at specific time assessments (binomial proportion tests, Holm corrected, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). Synergistic effect sizes for each time assessment are shown in electronic supplementary material, table S3.

bees, and *lower* dose bees consumed more than bees treated with *higher* doses (−25%, contrast test[DS]). There was no significant effect of TMX on sucrose consumption at any diet quality (GLM, $p > 0.3$; electronic supplementary material, table S4 and figure S1B).

(c) Sublethal doses of neonicotinoids reduced glucose and trehalose haemolymph levels

Glucose and trehalose haemolymph levels were only assessed on bees fed the *ad libitum*-quantity diet, because insufficient haemolymph was extractable from bees that were only fed the *limited*-quantity diet (10 µl). The haemolymph was extracted 2 h after the neonicotinoid exposure. There was a significant effect of CLO on glucose ($p = 0.0092$) and trehalose ($p = 0.0021$) haemolymph levels when foragers were fed a diet of *rich* quality (50% sucrose) (GLM; electronic supplementary material, table S5 and figure S2A). Specifically, the haemolymph of *control* bees contained higher levels of glucose than bees fed the *higher* (+26%) and *lower* (+27%) CLO doses. *Control* bee haemolymph also contained higher levels of trehalose than the haemolymph of bees fed the CLO *higher* dose (+26%, contrast test[DS]).

Likewise, there was a significant effect of TMX on glucose ($p = 0.0122$) haemolymph levels when foragers were fed diets of *rich* quality (GLM; electronic supplementary material, table S5 and figure S2B). Specifically, *control* bee haemolymph contained higher levels of glucose than that of bees exposed to *lower* (+55%) and *higher* (+60%) TMX doses (contrast test[DS]).

(d) Effects of nutritional deficits on pesticide-free bees

(i) Nutritional deficits decreased the survival of pesticide-free bees

As expected, the survival of pesticide-free bees fed the *limited*-quantity diet was significantly lower than the that of pesticide-free bees fed the *ad libitum* diet (Kaplan–Meier, $\chi^2 = 762.32$, d.f. = 1, $p < 0.0001$).

There was a significant effect of diet quality on the survival of pesticide-free foragers fed *ad libitum* (Kaplan–Meier, $p < 0.0001$; electronic supplementary material, figure S3A and table S6). Specifically, foragers fed lower-quality diets had a significantly shorter survival (Kaplan–Meier[DS], d.f. = 1, $p < 0.0001$; *poor* versus *intermediate*: $\chi^2 = 35.62$; *poor* versus *rich*: $\chi^2 = 100.16$; *intermediate* versus *rich*: $\chi^2 = 41.43$; electronic supplementary material, figure S3A).

There was a significant effect of diet quality on the survival of pesticide-free foragers fed *limited*-quantity diets (Kaplan–Meier, $p < 0.0001$; electronic supplementary material, figure S3B and table S6). Specifically, bees fed lower-quality diets had significantly reduced survival (Kaplan–Meier, d.f. = 1; *poor* versus *rich*: $\chi^2 = 5.45$, $p = 0.0196$; *no nutrients* versus *rich*: $\chi^2 = 37.30$, $p < 0.0001$; *no nutrients* versus *poor*: $\chi^2 = 9.02$, $p = 0.0027$; electronic supplementary material, figure S3B).

(ii) Lower-quality diets reduced glucose and trehalose levels in the haemolymph

In pesticide-free foragers, there was a significant effect of diet quality on glucose (GLM, $\chi_{7,2} = 22.42$, $p < 0.0001$) and trehalose (GLM, $\chi_{7,2} = 37.30$, $p < 0.0001$) levels (electronic supplementary material, figure S3E,F). As expected, forager haemolymph of bees fed *rich* diets contained significantly higher levels of both glucose and trehalose than those fed *intermediate* (+49% and +23%, respectively) and *poor* (+68% and +48%) diets (contrast test[DS]).

(iii) Diet quality influenced sucrose consumption

There was a significant effect of diet quality on sucrose consumption of pesticide-free foragers (GLMs, $\chi_{7,2} = 171.09$, $p < 0.0001$; electronic supplementary material, figure S3C). Foragers consumed significantly less sucrose when they were fed lower-quality diets (*rich* versus *poor*: −72%; *rich* versus *intermediate*: −33%; *intermediate* versus *poor*: −58%, contrast test[DS]; electronic supplementary material, figure S3C). There was no significant effect of diet quality on the volume of the sucrose solutions consumed daily by the foragers (GLMs, $\chi_{7,2} = 1.43$, $p = 0.488$; electronic supplementary material, figure S3D).

4. Discussion

One of the most common routes of honey bee pesticide exposure is via foragers collecting nectar and pollen. We demonstrate, for the first time, that nutritional stresses can act synergistically with a sublethal, field-realistic pesticide exposure and reduce honey bee survival. We also show that the exposure to nutritional and pesticide stressors impairs bee haemolymph energy levels and food consumption. Although prior research demonstrated that a good pollen diet can increase bee resistance to pesticides [21,23], and that food quality influences the effect of toxins on arthropod health [24–27], this is the first study to demonstrate the negative synergistic effects of sugar caloric restriction and pesticides in animals.

Bees that did not undergo nutritional stress were not significantly impaired by TMX or CLO. Forager survival was not significantly altered by any field-realistic doses of these neonicotinoids when they were fed optimal-quality and -quantity sugar diets (electronic supplementary material, table S1; figure 1*a*,*f*). This result also confirms that our doses were sublethal. However, bees fed a poor nutritional diet experienced detrimental synergistic effects, up to a 50% mortality increase when compared with the expected non-synergistic (additive) effects. Each neonicotinoid synergistically reduced survival of bees fed diets of low quality (32.5% and 15% sugar concentration) or quantity (*limited* 10 µl of sugar solution) (electronic supplementary material, tables S1 and S3; figures 1 and 2). This adverse synergistic effect of neonicotinoids and poor nutrition appeared rapidly after treatment (2 h; electronic supplementary material, table S2) and lasted up to 1 day (figure 2). Interestingly, starvation was less severe than pesticide exposure: bees survived longer when fed a pesticide-free diet containing no nutrients (pure water), when compared with bees that consumed a sugar diet of poor nutritional value, but containing a sublethal dose of pesticide (electronic supplementary material, table S1; figure 1*e*,*j*).

The combination of nutritional and neonicotinoid stressors also reduced food consumption (electronic supplementary material, figure S1). In all of our consumption experiments, bees were only fed pure sucrose solutions. Neonicotinoids were administered separately, prior to measuring consumption. Consumption was therefore not influenced by

the presence of neonicotinoids in the sucrose solutions [54]. When foragers were fed the richest-quality diets, their consumption was not significantly altered by any prior neonicotinoid exposure. However, all acute doses of CLO significantly reduced subsequent food consumption when bees were exposed to the poorest quality diet, suggesting that neonicotinoids alter foragers' energy metabolism or feeding behaviour.

What accounts for this change in feeding? TMX reduced forager motor functioning (acute exposure, 1.34 ng/bee; 2-day chronic exposure, $\text{range}_{\text{TMX daily doses}} = 1.42–3.48$ ng/bee d^{-1}) and food consumption (1 day of chronic exposure) [55]. The reduced motor functioning of neonicotinoid-treated bees may lead to decreased energy consumption and food intake [55]. Similarly, Kessler *et al*. [54] showed that chronic exposure to CLO (0.1–1 µM, 25–250 ppb) and TMX (0.1–1 µM, 29–292 ppb) reduced honey bee food consumption.

Neonicotinoid consumption also reduced sugar levels in the haemolymph of bees, measured 2 h after pesticide exposure (electronic supplementary material, figure S2). CLO exposure significantly decreased both trehalose and glucose titres. TMX significantly reduced glucose levels, although TMX did not alter sucrose consumption at any diet quality. TMX may have altered sugar metabolism. These alterations were only significant when bees were fed *ad libitum* diets of the richest quality. Bees fed *ad libitum* diets of poorer quality had very low haemolymph sugar levels (2 h after treatment) across all pesticide treatments. A likely explanation is that the poorer-quality diets could not fulfil bee nutritional requirements.

The food consumption and haemolymph sugar-level alterations caused by neonicotinoids can disrupt forager energy metabolism, which is important for honey bee colony health [56]. Specifically, the neonicotinoid, imidacloprid, inhibits mitochondria respiration and ATP synthesis [57], and increases brain oxidative metabolism [58]. Similarly, another pesticide (a triazole fungicide, myclobutanil) disrupts energy production through reduced mitochondrial regeneration and ATP production [59]. These energetic changes may have broader behavioural effects, interfering with thermoregulation [60], locomotion [55] and flight [61]. Flight is one of the most energy-intensive tasks [31], is fuelled by sugar oxidation [32], requires flight muscle thermoregulation [62] and is impaired by acute and chronic sublethal TMX exposures [61].

Although CLO and TMX elicited similar results, CLO exerted consistently stronger effects, which also appeared earlier after exposure, when compared with TMX. This may have occurred because TMX targets different nAChR subtypes with a lower affinity than CLO [16]. In fact, CLO ($LD_{50} =$ 4 ng/bee [47]) is more toxic than TMX ($LD_{50} = 5$ ng/bee [48]). Because approximately 36% of TMX degrades to its main metabolic by-product, CLO [16,63], the toxicity of TMX may be enhanced, to a degree, by its degradation to CLO. In cockroaches, the impairing effect of TMX on locomotion is correlated with its degradation to CLO [64].

As expected, richer sugar diets significantly increased survival (electronic supplementary material, figure S3A,B) and haemolymph energy levels (electronic supplementary material, figure S3E,F) in pesticide-free bees. Foragers consumed roughly the same maximum amounts of sucrose solution by volume because they consumed similar volumes of food across diet treatments (64 ± 1 µl/bee d^{-1}, mean of all pesticide-free diets; electronic supplementary material, figure S3D). Bees are evidently unable to compensate for a diet with low-sugar concentration by simply consuming a higher volume of sugar solution. In fact, although the mean sugar levels in the haemolymph of our bees were within the typical concentrations of glucose (2–20 µg µl^{-1}) and trehalose (2–40 µg µl^{-1}) [36,65–68], pesticide-free bees fed lower-quality diets had also lower haemolymph energy levels (electronic supplementary material, figure S3E,F).

Prior insect studies showed that nutritional deprivation impairs the immune functions of the mealworm beetle (*Tenebrio molitor* L.) [69] and decreases the longevity of the housefly (*Musca domestica* L.) [70]. Sugar scarcity affects the survival [71] and behaviour [72] of organisms with complex sociality, such as ants. Our results show that nutrient deprivation reduces the lifespan of honey bees, and also compromises their resistance and resilience (i.e. ability to recover from the acute sublethal exposure) to pesticides. These data highlight the fundamental importance of high-quality carbohydrate food for bees.

The behavioural and physiological impairments showed in our study probably compromise bee health, contributing to a broader variety of sublethal side effects (for reviews see [15,19]). Nutrition and pesticide stressors could trigger synergistic effects on other bee species. When compared with honey bees, bumblebees consume more food, while storing a lower quantity of it. They are, therefore, more dependent on available nectar sources than honey bees, while being similarly exposed to pesticides. In addition, bumblebee food consumption can be widely altered by chronic exposures to neonicotinoids, such as CLO (0.1–1 µM and 10 µg l^{-1}), TMX (1, 4, 39 and 98 µg kg^{-1}) and imidacloprid (0.001–1 µM and 0.8–125 µg l^{-1}) [54,73–75].

Current risk-assessment (RA) procedures used for testing chemicals do not fully take into account our current understanding of bee toxicology and health [22,26,76–78]. Our results raise further concerns by suggesting that the sugar diet regime typically used for RA toxicity tests may strongly influence pesticide toxicity. For example, the standard RA guideline for LD_{50} toxicity tests requires feeding bees with 50% (w/v) sucrose solutions *ad libitum* [46]. The results of these toxicity tests, obtained by feeding bees with an optimal nutritional diet, may underestimate the toxic effect that chemicals elicit on bees in the field, where foragers can be exposed to a combined nutritional stress (i.e. low-sugar nectar) [7,10,17,19]. Thus, the consequences of low-sugar nectar and neonicotinoid (TMX and CLO) exposure should be considered in assessing risks on insect pollinators. We suggest that RA procedures should test pesticide effects at various nutritional quality levels. More broadly, combined animal exposure to xenobiotic and nutritional stressors is a highly relevant ecological scenario that deserves greater attention.

Data accessibility. Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.kc680 [79].

Author contributions. S.T. conceived the experiments. S.T., J.C.N. and F.S. designed the experiments. S.T. and R.C. collected the data. S.T. and J.C.N. analysed the data. P.M. provided materials and reagents. S.T., J.C.N., F.S., R.C. and P.M. wrote and reviewed the manuscript.

Competing interests. We have no competing interests.

Funding. This study was partially funded by Council for Agricultural Research and Economics, Agriculture and Environment Research Centre and Alma Mater Studiorum, University of Bologna.

Acknowledgement. We are grateful to Luciano Cavani for the critical technical support for the haemolymph analysis. We thank Harmen Hendriksma for the suggestions provided on the manuscript. We thank the many students involved, especially Andrea Resconi, Michela Taccioli and Marco Gobbetti for their contribution in field and laboratory data collection.

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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 4^{th} and the 5^{th} 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 α = 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 α = 0.0170; *limited* quantity trial: k = 4, adjusted α = 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.

Diet quantity	Neonicotinoid	Diet quality (%)	N	Mortality (%)											
				Control			Lower			Higher			No nutrients		
				1 h	2 h	3 h	1 h	2 h	3 h	1 h	2 h	3 h	1 h	2 h	3 h
Ad libitum	TMX	Rich	270	1	1	1	0	0	0	0	0	0	1	6	16
		Intermediate	270	0	0	0	0	0	0	0	0	1			
		Poor	272	0	0	0	0	0	1	0	2	7			
	CLO	Rich	270	0	0	0	0	0	0	0	0	0			
		Intermediate	270	0	0	0	0	0	0	0	0	3			
		Poor	270	0	0	0	0	0	0	0	6	10			
Limited	TMX	Rich	405	0	0	0	0	0	0	0	1	4			
		Poor	407	0	1	3	0	1	4	0	3	19			
	CLO	Rich	405	0	0	0	0	0	0	0	0	3			
		Poor	406	1	1	3	0	0	3	0	4	15			

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

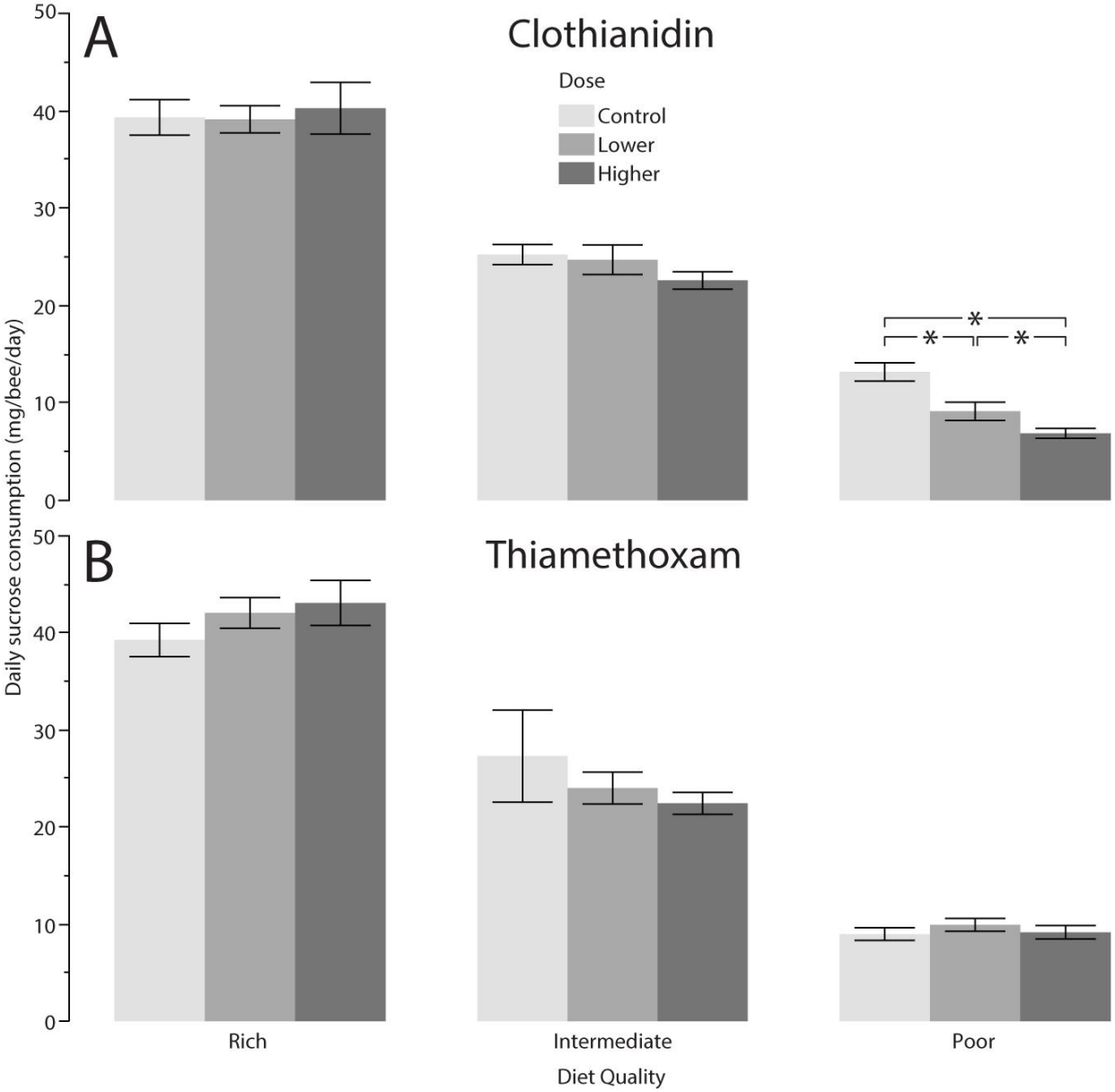
ESM table S5. Main effects of sublethal field-realistic dose of two neonicotinoids (CLO and TMX) on glucose and trehalose levels in forager hemolymph. Results are shown for each diet quality. Asterisks indicate significant effects of dose (GLMs).

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

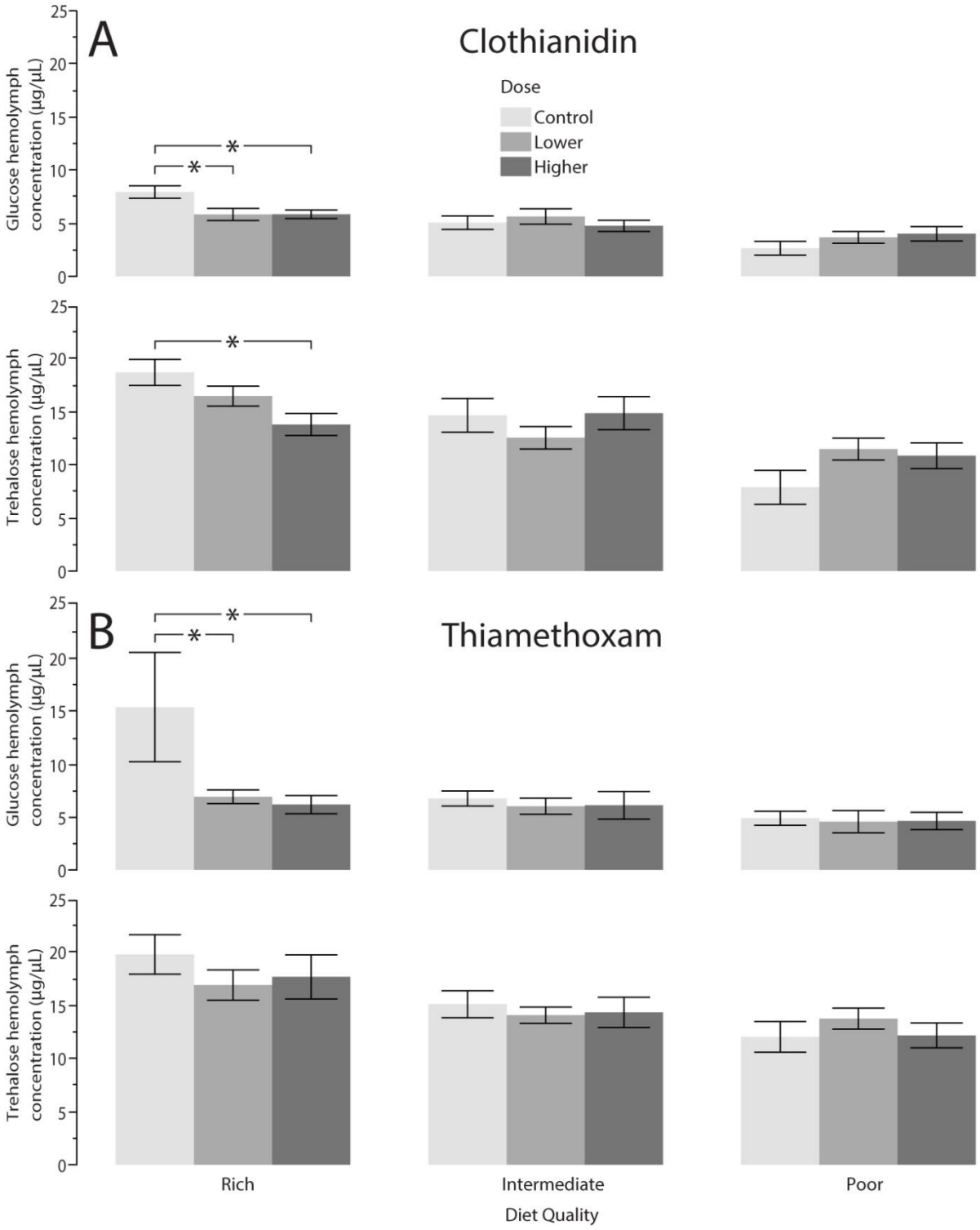
ESM table S6. Effects of diet quantity and quality on the survival of pesticide-free bees. We report the Lethal Time (LT) at which 25%, 50%, and 75% (LT25, LT50, and LT75) of bees died for each treatment. We tested the *no nutrients* diet (0% sucrose concentration) to include a scenario in which bees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

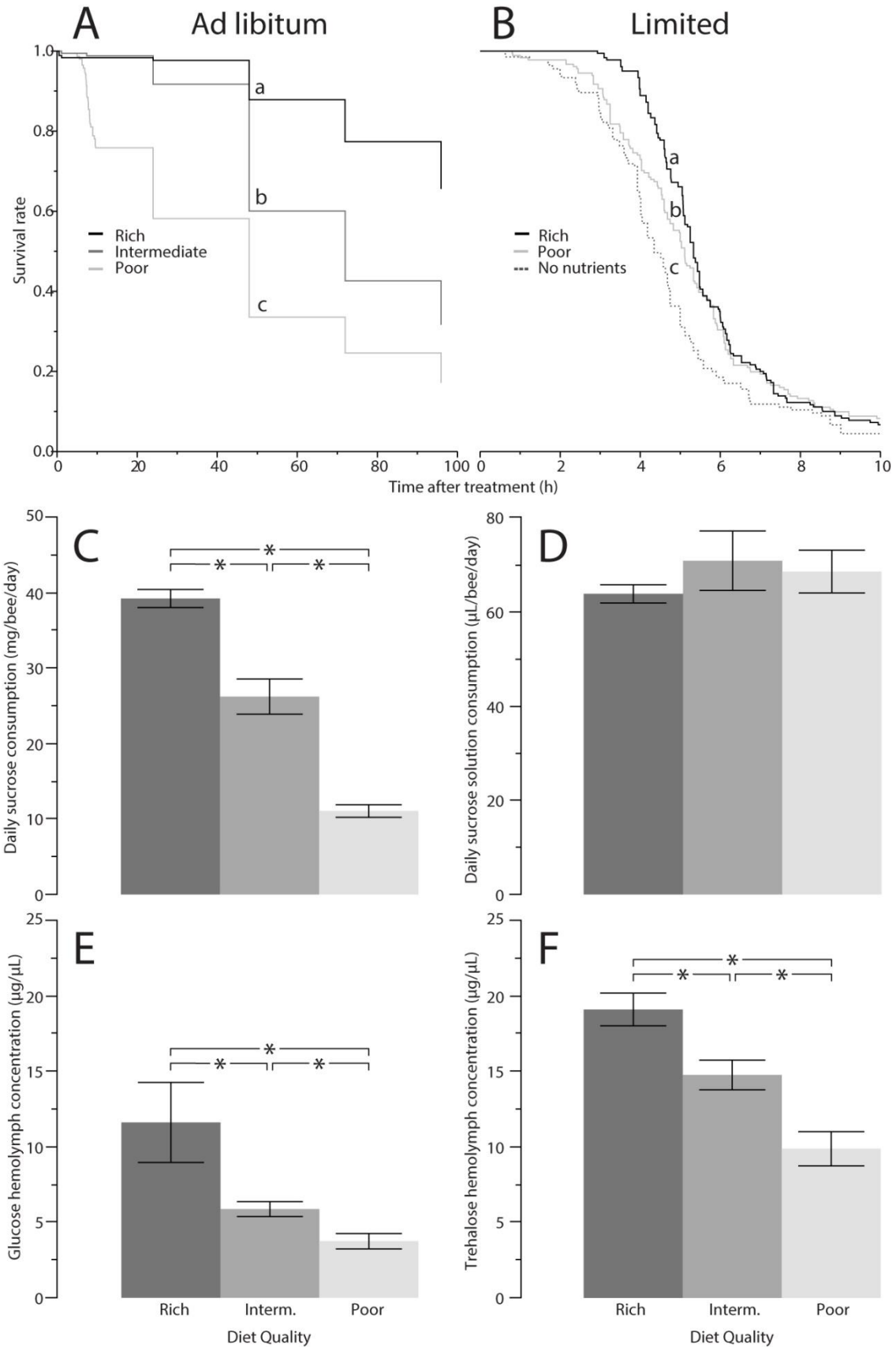
ESM FIGURES



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) $N_{Rich} = 72$, $N_{Intermediate} = 72$, $N_{Poor} = 72$).