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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 ± 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

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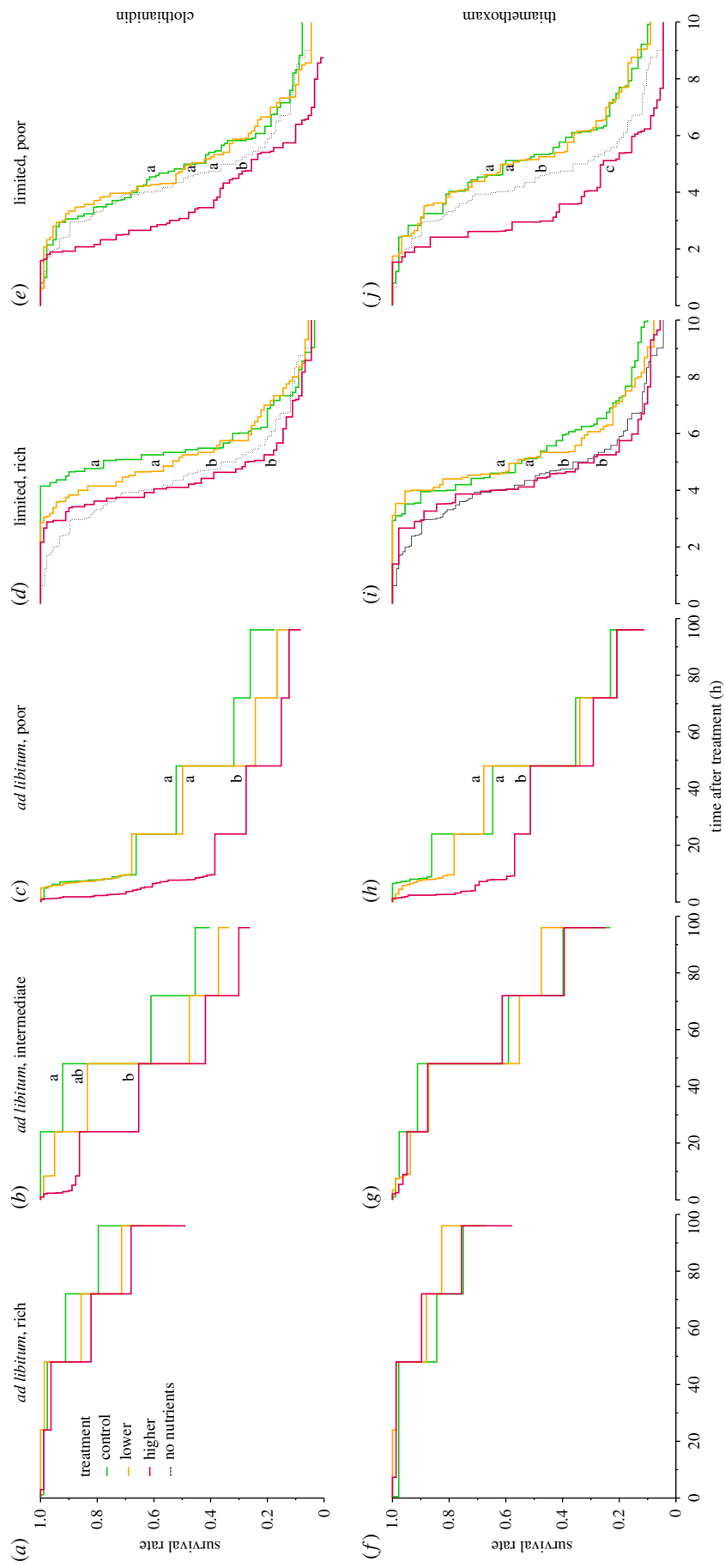
Figure 1. Survival of bees exposed to combined nutritional and pesticide stressors. We exposed bees to a control (green lines), lower (orange) or higher (red) field-realistic sublethal dose of CLO (*a–e*) or TMX (*f–j*). We fed bees *ad libitum* diets of *rich* (*a,f*), *intermediate* (*b,g*) or *poor* (*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.

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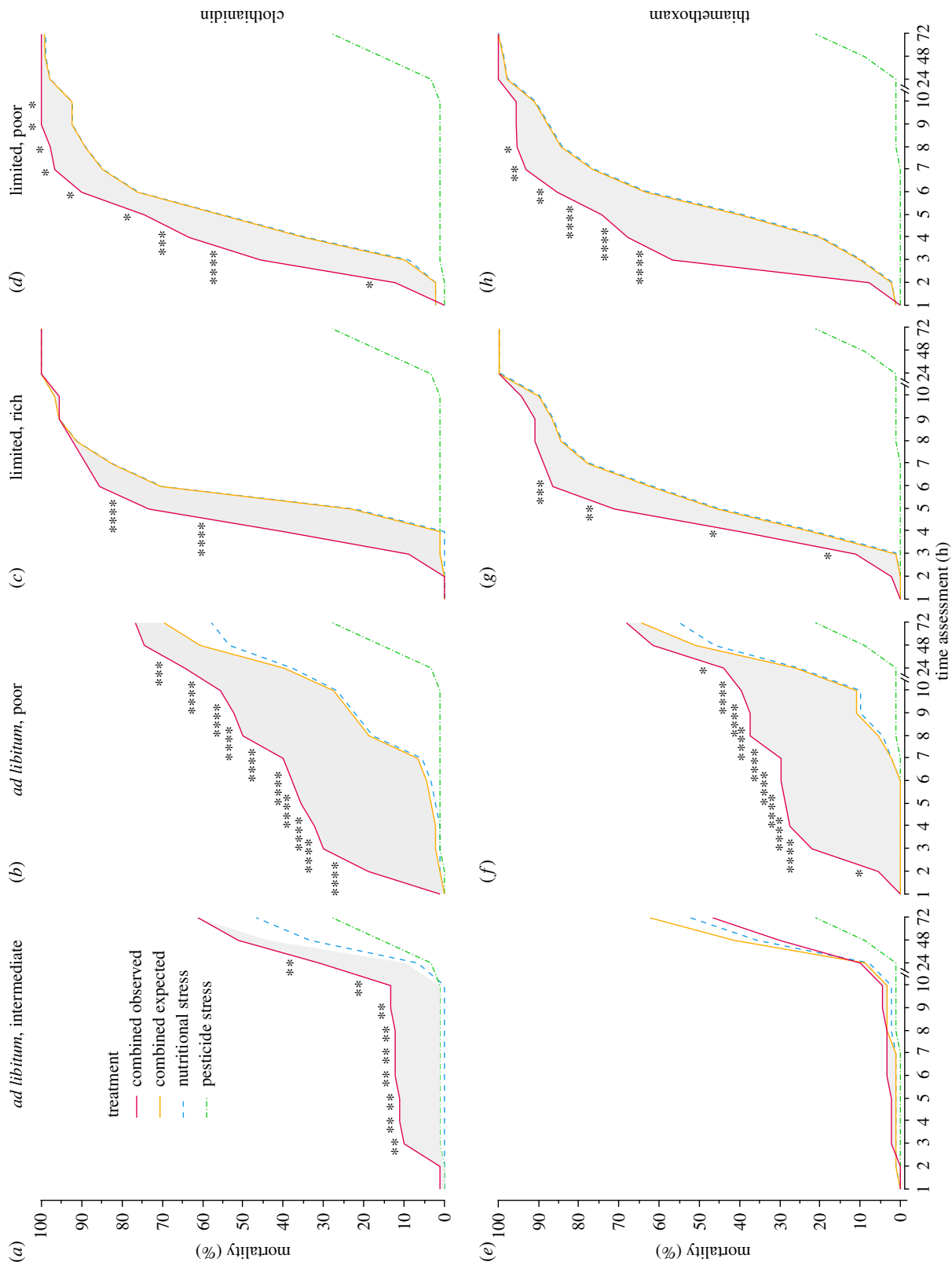
Figure 2. Synergistic effects of nutritional and neonicotinoid stressors on bee survival across time. We tested the individual and combined effects of each nutritional stress (treatment A, blue, dashed lines) and the higher neonicotinoid sublethal field-realistic dose of either CLO (a–d) or TMX (e–h) (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 combined treatment) at specific time assessments (binomial proportion tests, Holm corrected, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$). Asterisks indicate significant synergistic effects (i.e. significant difference between expected and observed mortality. 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 1a,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 1e,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

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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, $range_{TMX\ daily\ doses} = 1.42-3.48$ 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.

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