same procedure but were presented with a 10 µl droplet of sucrose solution (50°Brix). All workers were then placed back into their allotted Nicot cages and the sucrose syringe was returned. To enable the parasite to establish itself within the host the bees (both parasitized and unparasitized) were then left for 7 d (Schmid-Hempel and Schmid-Hempel 1993, Logan et al. 2005). 76 bumblebees died during this time period, but there was no difference in mortality between inoculated and uninoculated bees (see Supp Table S1 [online only]).

Thiamethoxam Exposure

Thiamethoxam PESTANAL analytical standard (100 µg) was purchased from Sigma-Aldrich and combined with 100 ml of acetone solution to produce the stock solution, which was subsequently combined with sucrose (50°Brix) to create the required dosages. The acute oral LD50 for thiamethoxam in *B. terrestris* has previously been determined to be 5 ng of active substance/bee (EFSA 2015) and we based our dosages on this (see Supp Table S1 [online only]).

Prior to being fed the relative thiamethoxam dose the sucrose syringes were removed from the Nicot cages and the bees starved for 3 h. Following this, the syringes were replaced with new ones with a 40 µl sucrose solution (50°Brix) containing the relevant thiamethoxam dosage. Bees were left for 4 h, after which the syringes were replaced with weighted syringes containing clean sucrose. Bees that had not consumed the entire dosages were removed from the experiment (n = 8, see Supp Table S1 [online only]).

Bees were left for 96 h and mortality was recorded at 4, 6, 8, 24, 48, 72, and 96 h, after being fed the thiamethoxam inoculation (OECD 2017). All bees that died during the experiment were frozen at −80°C.

Parasite Analysis

All bees were screened for *C. bombi* infection. Individual bees were dissected, and the hindgut was removed and placed into a 1.5 ml Eppendorf tube. 100 µl of 0.9% Ringer's solution was added and the hindgut was pulverised within the Ringer solution. The contents were then vortexed for 2 s. Uninoculated bees were checked for infection by placing 14 µl from each sample onto a microscope slide and analysing it for *C. bombi* cells under phase contrast at 400× magnification. No uninoculated bees were found to be infected.

For inoculated bees, we used a Neubauer improved haemocytometer to measure *C. bombi* intensity, and to count the number of *C. bombi* cells per µl. Inoculated bees that had no sign of an infection were removed from the analyses (n = 4).

Thorax width, as a proxy for body size, was measured using a Mitutuyo digital calliper, with all individuals measured three times to produce a mean measure of size.

Statistical Analysis

We used an information theoretic model selection approach for each test (except for determining the LD_{50} values [see below]). The initial model set contained all measured factors and was compared to all subsets of the full model, and a null model containing just the intercept and random factors. Models were selected based on Akaike weights derived from AICc values, and were included when they could not be rejected with a 95% certainty (this included cases in which the null model was accepted within the confidence set). When more than one model was present within the confidence set, model averaging was used (Burnham and Anderson 2002).

Following Ritz et al. (2015) we used a fitted dose-response model (*drc*) based on a log-logistic regression analysis to determine the LD_{50} values for bumblebees that were and were not inoculated with *C. bombi*. A mixed effect Cox model and a linear mixed effect model were used to determine if *C. bombi* influenced bumblebee mortality and sucrose consumption respectively. *C. bombi*, thiamethoxam dosage, and their interaction were included as fixed factors and bee size was included as a covariate. Colony of origin was included as a random factor. Parasite count was logged (log10) to improve model fit and analysed using a linear mixed effect model with thiamethoxam dosages, with bee size included as a covariant, and colony of origin included as a random factor.

We used the packages *drc*, *MuMin*, *lme4* & *coxme* (Bates et al. 2015, Ritz et al. 2015, Barton 2016, Therneau 2018).

Results

We found that the LD_{50} value for thiamethoxam was 6.63 ng when used in isolation compared with 6.82 ng per bumblebee when used in combination with the parasite *C. bombi*, suggesting no observed differences in mortality between infected and uninfected bumblebees (Fig. 1A and B, Coxme, *C. bombi*, Parameter Estimate (ES) = 0.10, Confidence Interval (CI) = −0.11 to 0.33). Bumblebee size had an effect on mortality, but the effect was not linear, with mortality risk increasing for both smaller and larger bees (Fig. 2, Coxme, size, PE = −0.35, CI = −0.60 to −0.11).

We found no effect of thiamethoxam or *C. bombi* inoculation on sucrose consumption (Fig. 3, Supp Table S2 and S3 [online only]). Interestingly, as thiamethoxam dose increased, this resulted in bumblebees having a higher intensity of *C. bombi* infection (Fig. 4A, lmer, dosage, PE = 0.0047, CI = 0.003–0.006). However, when subjects that died during the experiment were excluded from the analysis there was no effect of thiamethoxam dose on parasite intensity (Fig. 4B, lmer, dosage, PE = 0.001, CI = −0.01 to 0.01), suggesting no effect of thiamethoxam on *C. bombi* intensity at sub-lethal levels.

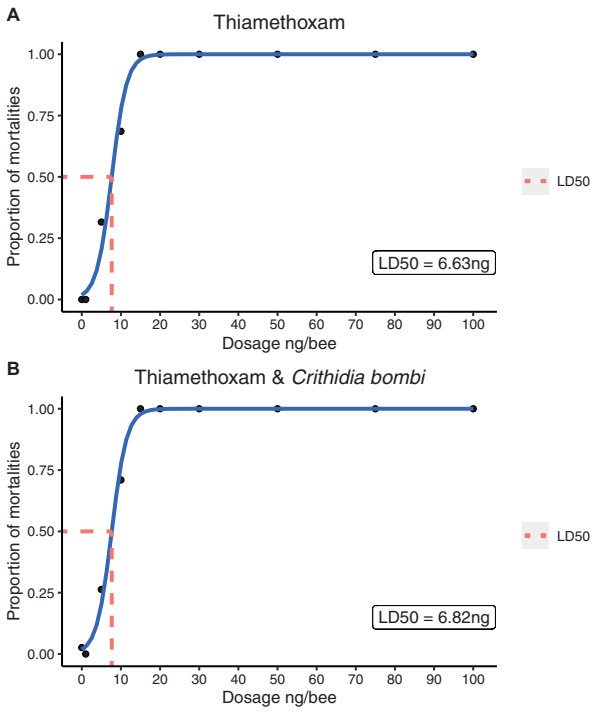

Fig. 1. Dose-dependent plots demonstrating the LD_{50} values for bees exposed to thiamethoxam in isolation (A) and bees inoculated with *C. bombi* and exposed to varying dosage of thiamethoxam (B). We found no difference in the LD_{50} between parasitized and unparasitized bees.

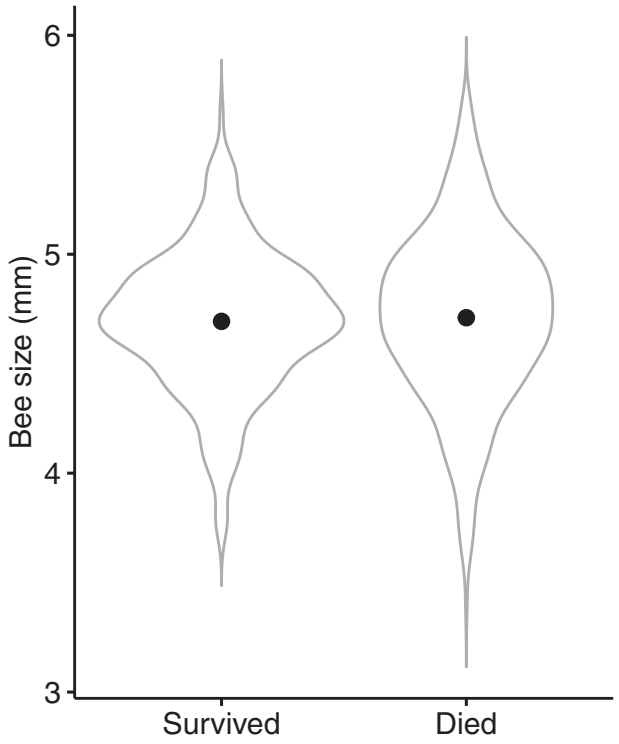

Fig. 2. Violin plots depicting the average size (mm) of bumblebees that either survived or died during the experiment (96 h). Mortality risk was higher for both smaller and larger bees.

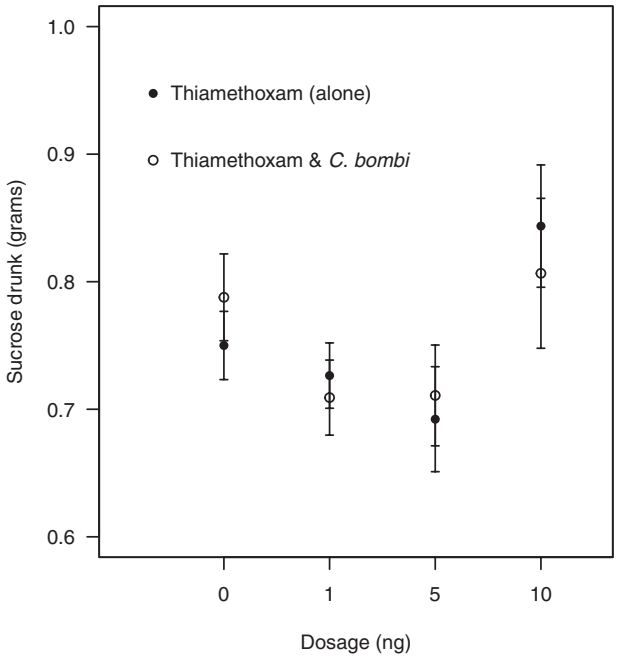

Fig. 3. The mean amount (grams) of sucrose drunk (±SE) over 96 h from parasitized and unparasitized bumblebees (*C. bombi*) acutely exposed to varying dosages of thiamethoxam. Subjects that did not survive the experiments were excluded from this analysis.

Discussion

Previous studies with bumblebees have shown that the LD_{50} of thiamethoxam is 5 ng of active ingredient per bumblebee (EFSA 2015),

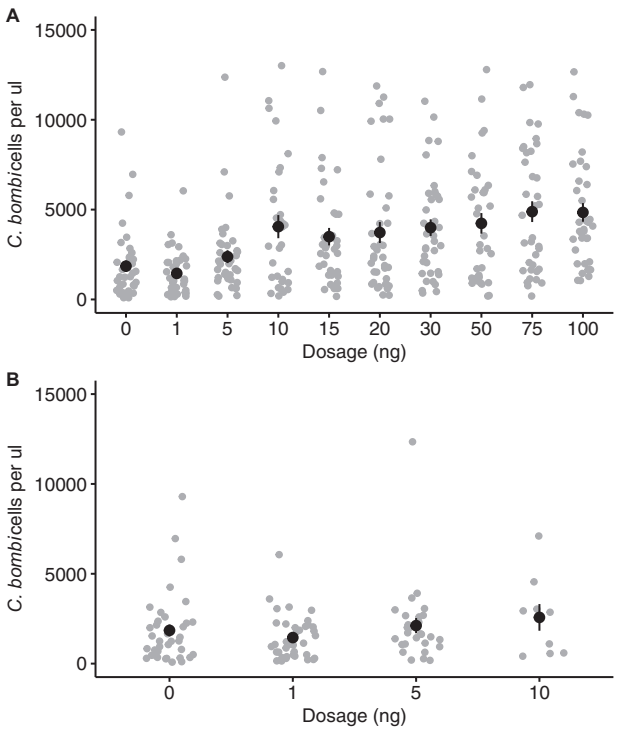

Fig. 4. The mean (±SE) number of *C. bombi* cells per µl found in the hindgut of all bumblebee workers from the experiment (A) and only bumblebees that survived until the end of the experiment (B).

and our results were similar (6.63 ng when exposed to thiamethoxam in isolation and 6.82 ng for bumblebees exposed to both thiamethoxam and *C. bombi*). This suggests that contrary to our original hypothesis, the parasite *C. bombi* had no impact on the LD_{50} of thiamethoxam on bumblebees (*B. terrestris*). This is surprising, as the effects of this parasite on bumblebees are context-dependent, and emerge most obviously when bees are exposed to other stressors (Brown et al. 2000, 2003; Yourth et al. 2008). Interestingly, and in contrast to previously observed results (Kessler et al. 2015, Arce et al. 2018) (but see [Muth et al. 2020]), we found no effect of thiamethoxam exposure on sucrose consumption. Finally, thiamethoxam exposure was seen to increase *C. bombi* intensity, but only at lethal dosages as there was no effect at sub-lethal levels. Our results demonstrate that methodologies currently used within the regulatory process can be modified to consider the interaction effects between multiple environmental stressors on wild bees.

We found no evidence of interaction effects between thiamethoxam and *C. bombi* on bumblebee mortality. This contrasts with previous studies that have shown that simultaneous exposure to both thiamethoxam and *C. bombi* can reduce bumblebee survival (Fauser-Misslin et al. 2014). However, Fauser-Misslin et al. (2014) assessed the impact of chronic, sub-lethal thiamethoxam concentrations over 9 wk on queen bumblebee survival, while here we used acute dosages, in a toxicity test with workers. Toxicity tests, such as LD_{50} experiments, are important in determining the lethal consequences of agrochemical use, but are not designed to detect more subtle, sub-lethal impacts of agrochemical exposure (Gill et al. 2012, Siviter et al. 2020b, Siviter et al. 2021b). While our modified LD_{50} protocol can be used to assess how parasites and agrochemicals interact at higher dosages, a failure to conduct sub-lethal assessments of chronic exposure in bumblebees alongside toxicity tests will clearly result in a failure to detect sub-lethal, but significant,