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Food Shortage Amplifies Negative Sublethal Impacts of Low-Level Exposure to the Neonicotinoid Insecticide Imidacloprid on Stream Mayfly Nymphs

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Abstract: Interactions of pesticides with biotic or anthropogenic stressors affecting stream invertebrates are still poorly understood. In a three-factor laboratory experiment, we investigated effects of the neonicotinoid imidacloprid, food availability, and population density on the New Zealand mayfly *Deleatidium* spp. (Leptophlebiidae). Larval mayflies (10 or 20 individuals) were exposed to environmentally realistic concentrations of imidacloprid (controls, 0.97 and 2.67 μg L^{-1}) for nine days following five days during which individuals were either starved or fed with stream algae. Imidacloprid exposure had severe lethal and sublethal effects on *Deleatidium*, with effects of the lower concentration occurring later in the experiment. The starvation period had delayed interactive effects, with prior starvation amplifying imidacloprid-induced increases in mayfly impairment (inability to swim or right themselves) and immobility (no signs of movement besides twitching appendages). Few studies have investigated interactions with other stressors that may worsen neonicotinoid impacts on non-target freshwater organisms, and experiments manipulating food availability or density-dependent processes are especially rare. Therefore, we encourage longer-term multiple-stressor experiments that build on our study, including mesocosm experiments involving realistic stream food webs.

Keywords: multiple stressors; pesticides; freshwater ecology; ecotoxicology; synergism; resource limitation; population density

1. Introduction

Neonicotinoids have become the most commonly used insecticides worldwide [1,2]. Because they persist in soil for many months and are highly water-soluble [3], they enter freshwaters predominantly by leaching into groundwater and surface runoff after rain, with the latter causing high-concentration pulses in surface waters [4–6]. Consequently, they have been frequently found in surface waters draining agricultural areas worldwide [4,7–11]. In North America and Europe, concentrations of imidacloprid—the most commonly detected neonicotinoid—often exceed safety benchmarks for freshwaters such as the United States acute and chronic invertebrate Aquatic Life Benchmarks (0.385 and 0.01 μg L^{-1}, respectively) [12].

Neonicotinoids are neuro-active compounds that are highly specific to invertebrates, particularly insects [13]. This means they have been perceived as relatively safe for vertebrate wildlife, human consumers of treated crops and operators applying these pesticides to crops or seeds. However, this specificity also means neonicotinoids pose a risk to non-target invertebrates. The pesticides bind to the postsynaptic nicotinic acetylcholine receptors (nAChRs), resulting in continuous nervous system stimulation, which is lethal at sufficient concentrations [14,15]. Larvae of aquatic insects are particularly

vulnerable to pesticides, with uptake occurring via gills and trachea during respiration, through feeding, and through the epidermis [16]. A number of recent studies have investigated toxic effects of neonicotinoids on freshwater invertebrates [5,16–21]. Among the organisms studied, mayflies (order *Ephemeroptera*) were the most sensitive to neonicotinoids [22,23]. This is a concerning finding, considering mayfly larvae are critical for supporting many aquatic and terrestrial food webs [5,6] and are important biological indicators of stream health [24,25].

Existing pesticide risk assessment programmes rely heavily on toxicity data produced by highly standardised laboratory experiments that can lack ecological realism [26–29]. To account for uncertainties related to the projection of toxicity assessments from benign laboratory conditions towards harsher field conditions and to predict regulatory acceptable concentrations (RACs), an assessment factor of 100 below the acute LC50 (the concentration lethal to 50% of the test organisms) has been established (see e.g., [30]). However, measured insecticide concentrations in surface waters often exceed their respective RACs, as observed, for example, in a meta-analysis of peer-reviewed literature on agricultural insecticide concentrations in EU surface waters [31]. Furthermore, detrimental impacts of pesticides on structure and/or biodiversity of agricultural streams have been observed frequently [32–34]. Indeed, an increasing number of recent studies have concluded that current pesticide regulations do not sufficiently protect the aquatic environment and that insecticides threaten aquatic biodiversity [5,6,31,35,36].

A basic requirement of valid toxicity tests is high survivorship of test organisms in control treatments, which means suboptimal conditions due to 'natural stressors' are usually avoided [18,37]. By contrast, in the real world these environmental variables fluctuate, often drastically, and optimal conditions for animals are rare [27]. Therefore, interactive effects of contaminants and natural stressors on non-target organisms are a possibility that should be more regularly considered in ecological risk assessment [38,39]. Abiotic factors such as water chemistry and temperature are increasingly investigated in order to improve our understanding of their effects on the toxicity of contaminants to aquatic organisms [40,41]. However, biotic factors such as food availability, competition, or predation are rarely included in such studies [18], and interactions between these natural stressors and contaminants such as pesticides remain largely unknown.

Laboratory studies typically provide test organisms with food ad libitum, whereas in realistic conditions nutritional resources are often restricted [42]. During development, animals can face transient periods of food shortage [43], causing them to lower their metabolic rate to increase their chance of surviving until more favourable conditions are reached [44]. Some studies suggest that the energy deficits resulting from resource limitations can increase an organism's sensitivity to a chemical [45,46]. Alternatively, the presence of a toxicant can impair foraging ability, possibly resulting in decreased survivorship [46]. A further environmental challenge for animals in natural systems is population density, which is often linked to food availability. Competitive processes have been shown to alter the outcome of pesticide exposure to invertebrates; for example, it has been suggested that competitive relationships may prolong the recovery of invertebrate populations following pulse exposure to pesticides [47], and that some populations may not recover at all in the presence of a less-sensitive competitor species [48]. Currently, there are no studies investigating the combined effects of food limitation, population density, and neonicotinoid exposure on freshwater invertebrates.

To reduce the abovementioned knowledge gaps, we employed a full factorial design to investigate the individual and combined effects of low-level exposure to the neonicotinoid imidacloprid, food availability, and density of individuals on the larvae of a common endemic New Zealand mayfly (*Deleatidium* spp.). *Deleatidium* are periphyton grazers that feed on the surfaces of rocks, including upper surfaces, and constitute an important food source for fish including introduced brown trout (*Salmo trutta*) [49]. We tested the following hypotheses:

1. Exposure to low concentrations of imidacloprid results in decreased survivorship, increased occurrence of impairment and immobility [6], and reduced moulting frequency in *Deleatidium*. Moulting is controlled by the endocrine and nervous systems; therefore, neurological disruption

due to imidacloprid may interfere with moulting [50], which has been observed in a previous 96-h imidacloprid exposure involving *Deleatidium* nymphs [41].

2. Due to the potentially suppressed metabolic rate of individuals [44], which may increase mayfly sensitivity to pesticides [42,45], a period of starvation prior to pesticide exposure causes amplified negative responses of survivorship, impairment, immobility, and moulting frequency.
3. A higher density of mayfly individuals intensifies the effect of the starvation period, with the resulting increase in competition for resources also amplifying the predicted adverse effects of imidacloprid on all four mayfly responses, similarly to above. Density-dependent increases in competition intensity are common in ecological systems [51].

2. Materials and Methods

2.1. Mayfly Food Supply

Our experiment was run in Austral winter in a temperature-controlled room at the University of Otago in Dunedin, New Zealand. Mayfly larvae were fed stream periphyton grown on unglazed terracotta tiles (10 × 10 × 1.4 cm). Roughly 300 tiles were placed in Lindsay Creek, a second-order stream, at a site in suburban Dunedin (45.8420 °S, 170.5408 °E). The site was concealed from public view, had *Deleatidium* mayflies present in its benthic invertebrate community, and was located in a small stream with a forested catchment that was unlikely to flood (causing bed disturbance) due to high rainfall. Additionally, because there is little urban influence and no agriculture upstream of the site (the stream drains a nature reserve clad in native forest culminating in Mt. Cargill at 680 m a.s.l.), it was unlikely that imidacloprid or any other pesticide was present in the stream. Tiles were exposed on 1 July 2016, two weeks prior to the start of the experiment, and collected on the morning of each day they were required.

2.2. Mayfly Collection

Roughly 750 *Deleatidium* larvae were collected on 15 July 2016 by electric fishing from a reach of Silver Stream (45.8096 °S, 170.4211 °E), a fourth-order stream near Dunedin. Electric fishing strongly increases invertebrate drift rates and is a fast, efficient method for collecting stream invertebrates. This technique has been used successfully in New Zealand and North America for ecological experiments where large numbers of live, unharmed invertebrate specimens were needed [49,52].

The stream reach is located in a native forest catchment without agriculture, thus the test organisms had no prior exposure to imidacloprid. A section of this reach measuring 75 m was repeatedly sampled using a pulsed DC backpack Electric Fishing Machine (Kainga EFM300; NIWA, Christchurch, New Zealand). *Deleatidium* larvae included a range of instars (sizes ranging 5–15 mm in length excluding tail filaments); late instar larvae with visible black wing pads were excluded due to an increased chance of emerging during the experiment.

Mayflies were returned to a climate-controlled room with a 16:8-h light:dark photoperiod and left overnight in aerated 4-L containers filled with stream water, to allow water temperature to adjust to the ambient temperature. Over the next 48 h, room temperature was gradually raised from 9 to 15 °C and then kept at 15 ± 1 °C for the entire experimental period (the same temperature as in a previous study on stream mayflies exposed to imidacloprid [53]). All mayflies were provided with periphyton tiles for food during this period to ensure they were not subjected to any food limitation prior to the experimental starvation period (see below). After the first 24 h, mayflies were transferred to aerated 4-L containers containing ASTM (American Society for Testing and Materials) artificial soft water (ASW) at climate-room temperature and then left to acclimate for a further 24 h. ASW consists of reconstituted deionized water containing known quantities of dissolved solutes (0.57 mM $NaHCO_3$, 0.17 mM $CaSO_4 \bullet 2H_2O$, 0.25 mM $MgSO_4$, and 0.03 mM KCl).

2.3. Experimental Design

Our experiment was conducted over 14 days in July 2016 (following the 2-day acclimation period), comprising a 5-day starvation period and 9 days of imidacloprid exposure. At the end of the acclimation period, 36 tiles with periphyton (see above) were collected from Lindsay Creek. Eighteen of these were scrubbed to remove all accrued algae so that half the experimental units were subjected to a starvation period while being provided with the same substratum as the non-starved group. Thirty-six 1.16-L glass containers measuring 19.9 × 14.4 × 6.3 cm (Keep'N'Box 116 cl; Luminarc, Arques, France) were filled with 500 mL ASW. One tile was added per container (tiles with and without algae were randomly allocated), and 20 mayflies were placed in each. Containers were placed randomly in an aeration tube setup using four air pumps (Aqua One Precision 7500; Aqua One, Ingleburn, NSW, Australia) to aerate all containers, and left for five days. This duration was chosen for the starvation period because in a previous related experiment, *Deleatidium* showed high survivorship (90%–100%) over 96 h plus an acclimation period, all with no feeding [41]. During previous pilot studies, we had also observed that the mayfly larvae showed clear signs of hunger after 48 h of no feeding: almost all individuals started feeding as soon as a tile with periphyton was placed into the container for the first time [54], a behaviour which was not observed when tiles were subsequently replaced. For these reasons, we were confident that most mayflies would survive the starvation period of five days while still experiencing some stress due to hunger.

In order to determine how the other two stressors—starvation and mayfly density—might interact with different levels of imidacloprid toxicity, our experiment required two distinctly different imidacloprid concentrations. Accordingly, we used one control concentration (0 μg L^{-1}), one 'lower' concentration expected to cause some sublethal adverse effects (in this case impairment; see below for a definition of this sublethal endpoint), and one 'higher' concentration expected to cause some mortality. These concentrations, 0.9 and 2.1 μg L^{-1} respectively, were determined from the results of two separate 28-day concentration-response experiments examining imidacloprid toxicity on *Deleatidium* larvae [55,56].

Following the starvation period, the three imidacloprid treatments were randomly allocated to the 36 experimental units, with 500 mL of solution in each container. Solutions were made using ASW and analytical standard grade imidacloprid (99.9% purity Imidacloprid PESTANAL®; Sigma-Aldrich, Castle Hill, NSW, Australia). A working imidacloprid stock solution (10 μg L^{-1}) was used to produce the treatment solutions. Control containers received 500 mL each of pesticide-free ASW. All tiles were replaced with a fresh tile (with periphyton food) from the stream, and all units were provided with periphyton food for the remainder of the experiment (the 9-day imidacloprid exposure period, see Section 2.4). To test the potential effects of mayfly density, containers were then manipulated so that half the units held 10 mayfly individuals and half held 20, by removing excess individuals from the '10' treatments and replacing any that had died in the '20' treatments while ensuring they were from the correct starvation treatment.

2.4. Determination of Mayfly Responses

Every three days during the imidacloprid exposure period (Days 3, 6, and 9), mayflies were observed and the numbers of dead, alive, impaired, and immobile individuals (out of those alive) and the mean number of moults per individual in each container were determined. In this experiment, we defined 'impairment' and 'immobility' as purely sublethal responses, to avoid confounding sublethal and lethal responses by combining the former with mayfly survivorship, and therefore only considered individuals that were still alive for these two sublethal responses. Impairment was defined as the inability of an individual to swim or to right itself. All individuals were gently lifted into the water column to test swimming ability—the natural response for a healthy *Deleatidium* larva is to swim toward a solid surface to cling to. If unable to swim, the individual was tested for righting ability by gently placing it ventral side up. An individual that was impaired could also be immobile, a more severe sublethal response. In order to test for immobility, an impaired individual was placed onto

a piece of stainless-steel mesh to test its ability to walk. If no sign of movement ability was evident besides some twitching of appendages, the individual was recorded as immobile.

Dead individuals and shed exuviae were removed from the containers daily. Periphyton tiles and solutions were replaced once during the pesticide exposure period, on Day 6, with tiles collected from the stream on the same morning. Also on Day 6, prior to replacing the tiles and solution, one 1000-µL water sample was taken from each treatment combination by sampling one of the three replicate containers at random (n = 12 in total) and freezing at −21 °C for later determination of the actual imidacloprid concentrations using the ELISA method (see Section 2.5). Three of the four water samples per imidacloprid addition treatment (0.9 and 2.1 µg L^{-1}; six in total) were randomly selected for ELISA testing.

2.5. Validation of Imidacloprid Concentrations

Concentrations of imidacloprid in the experimental units were verified using an enzyme-linked immunosorbent assay (ELISA) kit. This kit consists of a microplate with wells containing an antibody with a known binding efficiency for imidacloprid. Concentrations are determined by analysing the amount of imidacloprid that binds to these antibodies. A set of 'standards', with known imidacloprid concentrations, are analysed in the same way as the samples being tested. The kit (Imidacloprid ELISA Kit, 96 Test Microtiter Plate (Product No. 500800); ABRAXIS LLC, Warminster, PA, USA) had a detection range of 0.06 µg L^{-1} to 1.2 µg L^{-1}. According to the kit's instructions, concentrations around 0.4 µg L^{-1} (the middle of the calibration range) yield the most accurate results; therefore, samples were diluted to 0.4 µg L^{-1} prior to testing. Each sample was tested in either duplicates or triplicates. Immediately following the ELISA procedure, the microplate was analysed using a microplate photometer (FLUOstar Omega Microplate reader; BMG LABTECH, Allmendgruen, Ortenberg, Germany).

2.6. Data Analysis

Effects of the experimental manipulations on survivorship, impairment, immobility, and moulting in *Deleatidium* larvae were assessed using separate three-way ANOVAs in SPSS version 24 (IBM SPSS Statistics; IBM Corp., Armonk, NY, USA). The ANOVA model included three categorical fixed factors, 'imidacloprid concentration' (0, 0.9, and 2.1 µg L^{-1}), 'starved' (yes or no), and 'density' (10 or 20 individuals), plus all factor interactions. The resulting model was intercept (degrees of freedom 1) + imidacloprid concentration (d.f. 2) + starved (1) + density (1) + starved × concentration (2) + density × concentration (2) + starved × density (1) + starved × density × concentration (2) + error (24) (n = 36). For the survivorship response, total sample size (n) was 36 on all three sampling dates. For the sublethal responses impairment, immobility, and moulting, n differed depending on the experimental units remaining on a given date (n decreased with time as mayflies died; see Section 3.2). Because of this decrease, each sampling date was analysed individually for all mayfly response variables. ANOVAs were performed for all sampling dates where the study design was considered balanced enough. The significance level α for all tests was $p = 0.05$, and all response patterns summarised in the text were significant. For all significant main effects of imidacloprid concentration, pairwise comparisons were conducted with Tukey's HSD post-hoc tests. Standardised effect sizes (partial η^2 values, range 0–1; [57]) are presented for all p-values < 0.1 to allow our readers to evaluate the likely biological relevance of the results [58]. Effect sizes can be classified as: <0.10 very small, ≥0.10 small, ≥0.30 medium, and ≥0.50 large [59]. Because factor main effects can be difficult to interpret when significant interactions are present, for any results where an interaction had a larger effect size than the corresponding significant main effect(s), the interaction alone was interpreted [60].

3. Results

3.1. Achieved Imidacloprid Concentrations

Actual concentrations were (mean ± SE) 108 ± 5% (0.97 ± 0.05 μg L^{-1}) and 127 ± 10% (2.67 ± 0.2 μg L^{-1}) of the target concentrations of 0.9 and 2.1 μg L^{-1}, respectively. All figures, tables, and discussion are based on these achieved concentrations.

3.2. Sample Size

For mayfly survivorship, the full sample size of 36 experimental units remained across all three sampling dates, while sample sizes for the sublethal responses (impairment, immobility, and moulting frequency) decreased with time in some treatment combinations (Table 1). Because of this decrease, the experimental design became increasingly unbalanced from Day 6 onwards. Therefore, statistical results for the latter three responses are presented only for sampling dates where outputs were deemed robust enough to interpret. For illustrative purposes, response patterns for all three dates are shown for all four variables (Figures 1 and 2). However, for immobility and moulting frequency, only the statistically robust results of the ANOVAs for Days 3 and 6 are presented in tabular form (the data on Day 9 for these two responses contained many zero values and some missing values—see Figure 2).

Table 1. Numbers of replicate experimental units (with at least one alive animal) remaining for each treatment combination across sampling dates. Note that the response 'moulting frequency' was calculated using the sample size of the previous sampling date (three days earlier); therefore, sample sizes for this response variable are equal to that of the previous date.

Conc. (μg L^{-1})	Starved Y/N	Density	Day 3	Day 6	Day 9
0.0	N	10	3	3	3
0.0	N	20	3	3	3
0.0	Y	10	3	3	3
0.0	Y	20	3	3	3
0.97	N	10	3	3	2
0.97	N	20	3	3	3
0.97	Y	10	3	3	2
0.97	Y	20	3	3	3
2.67	N	10	3	2	0
2.67	N	20	3	3	1
2.67	Y	10	3	3	1
2.67	Y	20	3	3	2
Total	-	-	36	35	26

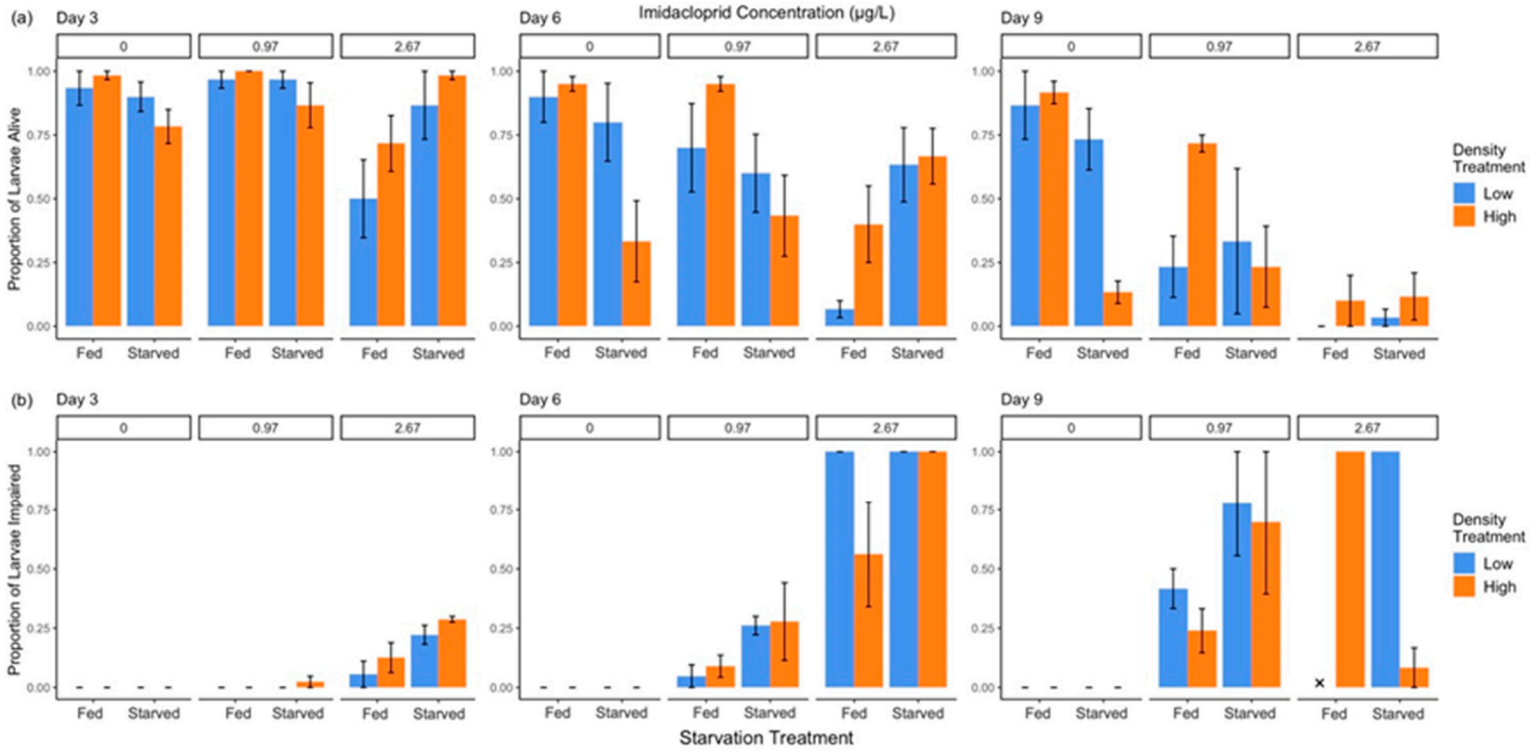

Figure 1. Responses of (**a**) survivorship and (**b**) impairment to the three imidacloprid treatments at the two mayfly nymph densities (10 and 20) and in the two starvation treatments (starved = starved for five days immediately prior to the imidacloprid exposure period). Bars represent means ± SEs. In cases where bars are missing, '×' represents a treatment group in which all mayflies had died in all replicates—i.e., no sublethal responses were measured—whereas '_' represents a zero value. See Tables 2 and 3 for statistical results.

3.3. Mayfly Responses

3.3.1. Survivorship

Deleatidium survivorship generally decreased in one or both imidacloprid addition treatments on all days of the imidacloprid exposure period (Figure 1a, Table 2). On Days 3 and 6, the main effect of imidacloprid on survivorship was overridden by a stronger starvation × imidacloprid interaction (see below). Effects of imidacloprid exposure increased with time. The largest effect of all treatment combinations on mayfly survival was the imidacloprid main effect on Day 9 (partial $\eta^2 = 0.67$), when survivorship decreased in a concentration-dependent manner.

Survivorship was also influenced by a starvation × imidacloprid interaction on all three dates (Table 2). The general pattern across all dates was that in treatments without imidacloprid addition, survivorship was lower in starvation treatments (Figure 1a). In the lower imidacloprid treatment, this pattern was also evident, whereas in the higher treatment the pattern was reversed (except for Day 9 when survivorship was generally low in the higher treatment).

Mayfly density alone had no effect on survivorship; however, on Day 9 there was a weak interaction between density and imidacloprid (Table 2). In treatments without imidacloprid addition, mean survivorship (across both starvation treatments) was lower in mayfly groups with 20 individuals than in those with 10 individuals, whereas this pattern was reversed in the two imidacloprid addition treatments (Figure 1a). Finally, on Days 6 and 9, there was an overall (in the control and lower imidacloprid treatments) negative effect of starvation on mayfly groups with 20 individuals, whereas starvation had little to no effect on groups with 10 individuals (starvation × density interaction).

Table 2. Results of three-way ANOVAs comparing the 'survivorship' response between experimental treatments. *p*-values < 0.05 are in bold text, and effect sizes (ES; partial η^2 values; range 0–1) are given in brackets where $p < 0.10$. Two interactions that overrode main effects of imidacloprid (due to a larger effect size) are underlined. Effect size categories are: 'trivial' < 0.1, 'small' > 0.1, 'medium' > 0.3, 'large' > 0.5 [59]. Treatment level rankings for significant main effects of imidacloprid are based on significant differences determined in pairwise comparisons using post-hoc tests. Imidacloprid concentrations in these rankings are in μg L^{-1}.

Sampling Day	Imidacloprid Concentration (IMI) *p* (ES) Ranking	Starvation *p* (ES)	Density *p*	Starved × IMI *p* (ES)	Density × IMI *p* (ES)	Starved × Density *p* (ES)	Starved × Density × IMI *p*
Day 3	**0.01** (0.32) 0.97 > 2.67	0.34	0.48	**0.001** (0.43)	0.14	0.16	0.96
Day 6	**0.007** (0.34) (0 = 0.97) > 2.67	0.27	0.94	**<0.001** (0.49)	0.11	**0.01** (0.25)	0.84
Day 9	**<0.001** (0.67) 0 > 0.097 > 2.67	**0.01** (0.27)	0.97	**0.03** (0.25)	**0.03** (0.25)	**0.01** (0.27)	0.15

3.3.2. Impairment

The proportion of impaired mayfly nymphs (out of those alive) in treatments without added imidacloprid was zero on all three sampling dates, and imidacloprid addition strongly increased impairment on all dates (Figure 1b, Table 3). On Day 3, imidacloprid only increased *Deleatidium* impairment at the highest concentration (2.67 μg L^{-1}), whereas on Day 6, impairment increased in a concentration-dependent manner. By Day 9 (when few surviving mayflies remained, see Figure 1a), impairment was similar at 0.97 μg L^{-1} and at 2.67 μg L^{-1}.

Starvation weakly increased impairment on Day 6, and on Day 3 there was a moderate main effect of starvation but a stronger starvation × concentration interaction overrode it (Table 3). On Day 3, starved mayflies showed greater impairment than non-starved mayflies, but only in the higher imidacloprid treatment (Figure 1b). A strong starvation × concentration interaction also occurred

on Day 9. This effect was similar to that observed on Day 3, except that on Day 9 the increase in impairment with starvation occurred only in the lower imidacloprid treatment (note that the number of experimental units in the higher imidacloprid treatment had decreased considerably by Day 9 due to many mayfly deaths, see Table 1).

Also on Day 9, mayfly density had a moderate main effect on impairment, but a slightly stronger interaction between density and concentration overrode this main effect (Table 3). Impairment was somewhat higher in containers with 10 individuals than in those with 20, but only in the lower imidacloprid treatment (Figure 1b). In the higher imidacloprid treatment, the much smaller, unequal sample sizes (with one treatment combination missing completely) did not allow reliable interpretation of this interaction.

Table 3. Results of three-way ANOVAs comparing the 'impairment' response between experimental treatments. p-values < 0.05 are in bold text, and effect sizes (ES; partial η^2 values; range 0–1) are given in brackets where $p < 0.10$. Two interactions that overrode main effects of starvation or density (due to a larger effect size) are underlined. See Table 2 for further details.

Sampling Day	Imidacloprid Concentration p (ES) Ranking	Starvation p (ES)	Density p (ES)	Starved × IMI p (ES)	Density × IMI p (ES)	Starved × Density p	Starved × Density × IMI p
Day 3	**<0.001** (0.80) (0 = 0.97) < 2.67	**0.001** (0.36)	0.113	**<0.001** (0.47)	0.21	0.84	0.93
Day 6	**<0.001** (0.91) 0 < 0.97 < 2.67	**0.01** (0.26)	0.22	0.16	0.12	0.18	0.13
Day 9	**<0.001** (0.76) 0 < (0.97 = 2.67)	0.40	**0.01** (0.35)	**0.002** (0.56)	**0.03** (0.38)	0.80	0.80

3.3.3. Immobility and Moulting

For these two sublethal responses, only the results of the ANOVAs for Days 3 and 6 were statistically robust, due to the reduced, strongly unequal sample sizes on Day 9 (see Section 3.2). As for mayfly impairment, the proportion of immobile nymphs (out of those alive) in treatments without added imidacloprid was zero on all sampling dates (Figure 2a). On Day 3, immobility was either zero or very low and unaffected by the experimental manipulations (Table 4). On Day 6, immobility increased strongly at the higher imidacloprid concentration (Table 4, Figure 2a).

A moderate effect of starvation, also on Day 6, was overridden by a slightly larger starvation × concentration interaction (Table 4). In this interaction, the increase in immobility in treatments with added imidacloprid compared to controls was amplified by starvation, especially at the higher imidacloprid concentration.

In contrast to the other two sublethal responses, moulting frequency of mayfly nymphs (always calculated using the sample size of the collection date three days earlier, see Table 1) was unaffected by all manipulated factors and their interactions (Table 4, Figure 2b).

Table 4. Results of three-way ANOVAs comparing responses between experimental treatments for 'immobility' (grey) and 'moulting frequency' (white). p-values < 0.05 are in bold text, and effect sizes (ES; partial η^2 values; range 0–1) are given in brackets where $p < 0.10$. One interaction that overrode a main effect of starvation (due to a larger effect size) is underlined. See Table 2 for further details.

Sampling Day	Imidacloprid Concentration p (ES) Ranking	Starvation p (ES)	Density p	Starved × IMI p (ES)	Density × IMI p	Starved × Density p	Starved × Density × IMI p
Day 3	0.16	0.87	0.17	0.97	0.16	0.87	0.97
	0.60	0.61	0.50	0.76	0.47	0.87	0.38
Day 6	**<0.001** (0.54) (0 = 0.97) < 2.67	**0.004** (0.31)	0.81	**0.01** (0.34)	0.32	0.16	0.58
	0.56	0.54	0.78	0.93	0.78	0.31	0.29

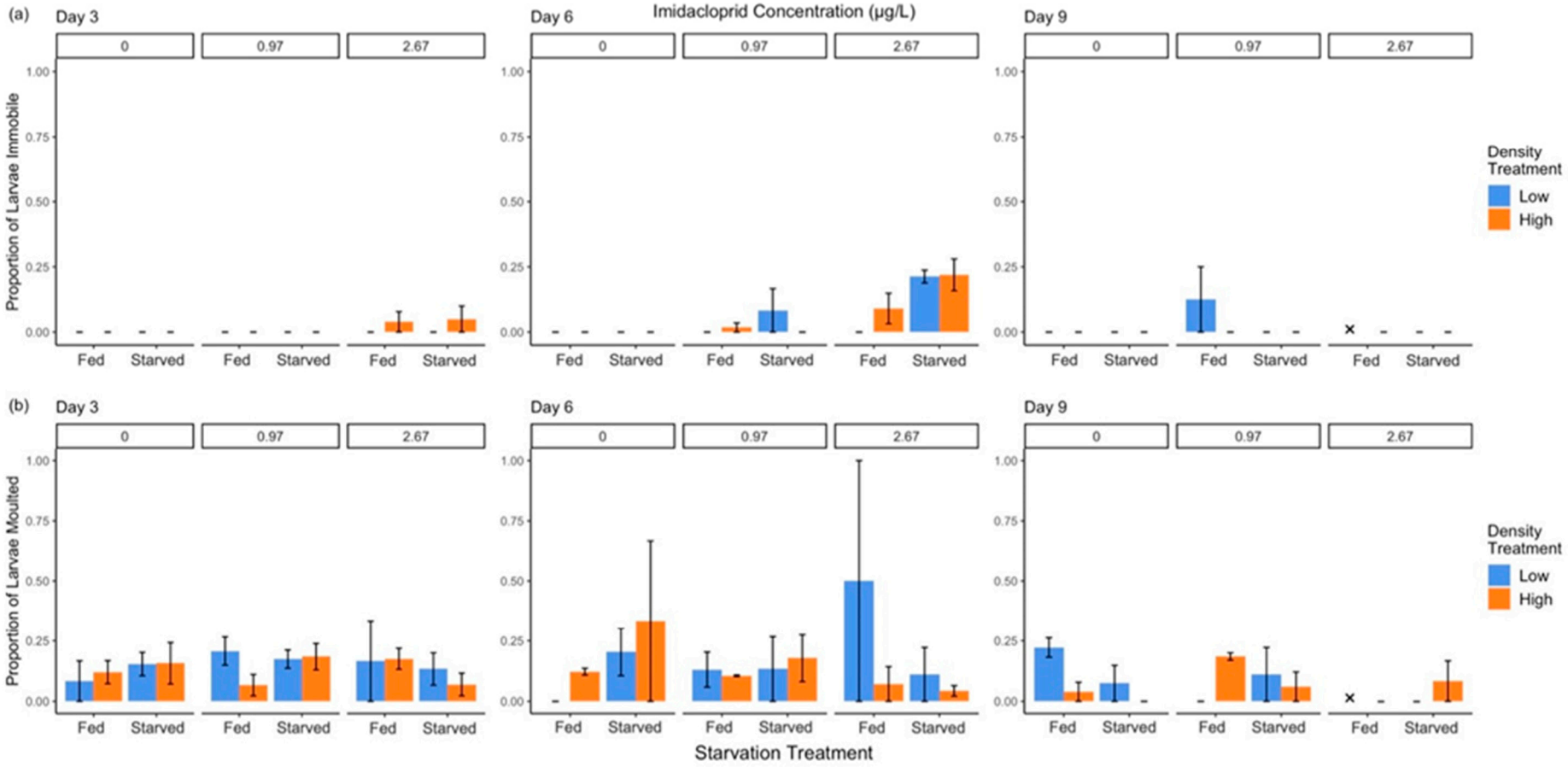

Figure 2. Responses of (**a**) immobility and (**b**) moulting frequency to the three imidacloprid treatments at the two mayfly nymph densities (10 and 20) and in the two starvation treatments (Starved = starved for five days immediately prior to the imidacloprid exposure period). Bars represent means ± SEs. In cases where bars are missing, '×' represents a treatment group in which all mayflies had died in all replicates—i.e., no sublethal responses were measured—whereas '_' represents a zero value. See Table 4 for statistical results.

4. Discussion

4.1. Imidacloprid Effects on Mayflies

Our experiment investigated the individual and interactive effects of exposure to field-realistic concentrations of imidacloprid, a starvation period, and population density on nymphs of the mayfly *Deleatidium*. Overall, imidacloprid exposure strongly reduced mayfly survivorship and strongly increased impairment, supporting our first hypothesis. Imidacloprid also resulted in strongly increased levels of mayfly immobility, again as predicted, although only on one sampling date (Day 6), most likely because all individuals immobilized on Day 6 had died by Day 9. Our higher imidacloprid concentration (2.67 μg L^{-1}) had relatively immediate effects on both survivorship and impairment, whereas the lower concentration (0.97 μg L^{-1}) had little effect immediately but 'caught up' with the higher concentration by the ninth day of exposure. The delayed effects on mayfly responses at our lower concentration are not unexpected because the toxicity of neonicotinoids is time-dependent [6,61] and the blockage of nAChRs in the invertebrate central nervous system may be largely irreversible [62,63] (see Section 4.4 for further discussion of this point).

4.2. Starvation Effects and Interactions with Imidacloprid

The importance of strengthening the integration of ecological principles into the field of ecotoxicology is increasingly being recognized [27,64–66]. Our study was designed to make a step towards this goal by determining the effects of exposure to imidacloprid in combination with two biotic 'natural stressors' commonly affecting mayfly larvae in streams. Robust main effects of the prior 5-day starvation period on Day 6 (increased impairment) and Day 9 (reduced survivorship) of the pesticide exposure period indicated that starvation had delayed effects on both mayfly responses. In another multiple-stressor experiment involving insecticide exposure with a prior starvation period, Dinh, Janssens, and Stoks [67] found that starvation had both immediate and delayed negative sublethal effects on the growth rate and physiology of larvae of the European damselfly *Coenagrion puella*. A six-day starvation period (prior to seven days of exposure to the neuro-active insecticide chlorpyrifos) reduced growth rate and various proxies of insect immune system activity, indicating some suppression of metabolic rate and immune function, both during the starvation period and continuing into the pesticide exposure period. This finding parallels our own results. Nevertheless, starvation did not interact with the pesticide to reduce *C. puella* survivorship, which was only significantly reduced when individuals were exposed to chlorpyrifos and a combination of prior starvation and a prior heat wave (three-way interaction) [67]. In our study, interactions between starvation and imidacloprid concentration occurred on several sampling dates for survivorship, impairment and immobility. However, in the case of survivorship, this was not usually because the prior starvation period amplified the adverse effects of the subsequent imidacloprid exposure, as we had predicted in our second hypothesis. Instead, such an amplified negative effect of starvation occurred only at the lower imidacloprid concentration, whereas starvation appeared to have a positive effect on survivorship at the higher imidacloprid concentration. By contrast, the sublethal mayfly responses largely supported our hypothesis. Both mayfly impairment (on Day 3) and immobility (on Day 6) were amplified in starvation treatments in the earlier stages of the imidacloprid exposure period, and these effects were ultimately drowned out by the more severe lethal impacts of imidacloprid on Day 9.

Experiments investigating the potential interactions between nutritional conditions of non-target aquatic organisms and pesticides are scarce [39]. However, a 2013 study on the damselfly *Enallagma cyathigerum* by Janssens and Stoks [68] revealed that nutritional conditions can influence the outcome of exposure to chlorpyrifos. The damselfly larvae responded to the pesticide by accelerating their development—possibly an adaptive response to shorten larval lifespan—but could do so only under optimal nutritional conditions. Immune function in invertebrates is known to be variable depending not only on food availability but also a range of other fluctuating abiotic factors such as salinity and temperature [69,70], and it is likely that there are physiological trade-offs involved which may alter the

outcome of exposure to contaminants such as neonicotinoids. Consequently, while support for our hypothesis regarding adverse synergistic interactions between starvation and imidacloprid exposure was evident only for sublethal responses, our findings along with evidence from previous studies suggest that further research on this topic is warranted.

4.3. Mayfly Density Effects and Interactions with Starvation or Imidacloprid

Mayfly population density (10 or 20 individuals per container) had no robust factor main effects on any of the studied mayfly responses, but significant interactions of density with starvation occurred for survivorship on Days 6 and 9. On these days, survivorship in the lower imidacloprid treatment and especially in controls was overall lower in starved treatments. However, this effect was more pronounced in starved containers with 20 individuals, particularly in controls on Day 9 (where survivorship was only about 10%, compared to about 70% in starved containers with 10 individuals). This response pattern suggests that mayfly larvae found it harder to recover from the starvation period at the higher population density, perhaps due to increased competition for the periphyton resources provided on the pre-colonised tiles. Interestingly, this potential consequence of intra-specific competition did not become evident until six days after the end of the five-day starvation period. Furthermore, there were weak to moderate interactions between mayfly density and imidacloprid concentration on Day 9 for survivorship and impairment. However, the generally low survivorship at the higher imidacloprid concentration and the small, unequal sample sizes for impairment on this day prevented biologically meaningful interpretations of these two interactions.

Currently, no other studies have investigated the possible combined effects of population density and neonicotinoids on non-target freshwater invertebrates. The few existing studies investigating density-dependent or competitive processes and their interactions with pollutants [47,48,71,72] involved interspecific interactions or looked at very different responses such as individuals' longevity. Consequently, there is little to compare these findings of our study to. In fact, population density of test organisms was not mentioned in two recent reviews of pollutant interactions with natural variables or stressors [38,39].

In our experiment, the higher mayfly population density led to increased mayfly mortality when combined with a preceding starvation period (as discussed above), whereas population density had no effects on the studied sublethal mayfly responses. It is worth noting that density of *Deleatidium* larvae in the bed substrata of New Zealand streams can exceed 6000 individuals per m^2 [73]; this is more than 8.5 times the density in our 20-nymph treatment (which is equivalent to 698 individuals per m^2). Thus, intra-specific competition for periphyton food resources among *Deleatidium* individuals during periods of food shortage could be considerable in real streams.

4.4. Which Are the Most Informative Sublethal Mayfly Responses?

While quite a few studies have now assessed the toxicity of imidacloprid to mayfly larvae [22,23,41,53,74–79], the most reliable indicators of sublethal toxic effects on these particular organisms have not been well established. In our study, 'impairment' and 'immobility' were treated as distinct, purely sublethal responses, with neither response including mortality and impairment occurring at lower concentrations than immobility. A recent study on the North American mayfly *Isonychia bicolor* also documented several sublethal responses to imidacloprid that occurred prior to total immobilization, including muscle spasms and lethargy [74]. While the blockage of nAChRs in the invertebrate central nervous system by neonicotinoids is widely regarded as irreversible [63,64], there is some recent evidence of the potential for reversible binding, with recovery of two freshwater invertebrates (including the North American mayfly *Neocloeon triangulifer*) from an immobilized state observed following one short-term pulse of imidacloprid [76]. For mayflies at least, it therefore seems that 'impairment' as defined in our study (without including mortality) is a crucial sublethal endpoint to consider, as such impairments most likely alter an individual's ability to feed, evade predators, and

therefore survive in real stream communities. Thus, studies that do not address sublethal endpoints, such as impairment, may underestimate the true toxicity of imidacloprid [74].

None of our three experimental factors or their interactions affected *Deleatidium* moulting frequency. This was generally very low, and statistical analysis was possible only on Days 3 and 6 of the pesticide exposure period. These results contrast with those from the 96-h experiment by Macaulay et al. [41], the only other existing study involving *Deleatidium* nymphs and imidacloprid. In that study, *Deleatidium* moulting frequency was generally much higher than in the present study, and imidacloprid exposure decreased moulting frequency. The lack of consistency across these studies, in spite of both being run during Austral winter, suggests that moulting frequency may not be a reliable sublethal response for *Deleatidium* nymphs, although it could be for other mayfly taxa [80,81].

4.5. Limitations of Our Study

We acknowledge that, compared to the relatively few existing mesocosm experiments in which entire stream communities were exposed to pesticides combined with other stressors, e.g., [66,82,83], our multiple-stressor laboratory study has low environmental realism, which limits the extrapolation of our findings to real streams. Nevertheless, similar to the laboratory experiment on damselfly larvae by Dinh et al. [67] in which prior exposure to a simulated heat wave under food limitation made the insecticide chlorpyrifos more lethal, our experiment provides a 'proof-of-principle' that synergistic interactions between a neonicotinoid and food limitation can worsen sublethal impacts on stream insects. Our findings warrant further tests of this 'proof-of-principle' in more field-realistic experiments.

Second, our selected two imidacloprid concentrations were successful in producing different lethal and sublethal mayfly responses, but the higher achieved concentration (2.67 μg L^{-1}) caused rather severe effects on some of the responses from Day 6 of the pesticide exposure period onwards. This potentially made detection of interactions between the three experimental factors more difficult because the generally strong effect of imidacloprid exposure may have hidden weaker effects of the other two factors.

Finally, the concentration ranges of neonicotinoids in New Zealand's freshwaters are still poorly known. Neonicotinoids are widely applied in agriculture and horticulture [84] and have recently been detected in agricultural streams [85] and in groundwater [86], and several other pesticides have been found in sheep/beef farming streams [87,88]. However, because the first stream survey in New Zealand for neonicotinoids was conducted during a long drought and the limited number of sites sampled may not have included any sites with high pesticide use in their catchments, the concentrations measured are probably serious underestimates of the concentrations that can occur [85]. Consequently, our experimental imidacloprid concentrations can be mainly compared to the commonly detected concentrations outside New Zealand. A 2016 meta-analysis of neonicotinoid residues in freshwaters across 11 countries by Sánchez-Bayo et al. [6] found an average detected imidacloprid concentration of 0.73 μg L^{-1}, with maximum concentrations reaching up to 320 μg L^{-1}. Of the 27 surveys testing for imidacloprid in this meta-analysis, the average concentrations exceeded our low concentration of 0.97 μg L^{-1} in six studies, and the highest concentrations detected exceeded both our treatment concentrations in 11 studies. In another survey of streams around Sydney, Australia, imidacloprid concentrations were mostly between 0.05–1.0 μg L^{-1} but reached up to 4.6 μg L^{-1} [4]. Based on these data, the imidacloprid concentrations used in our study (0.97 and 2.67 μg L^{-1}) seem likely to occur in New Zealand streams draining agricultural or urban landscapes to which neonicotinoids are applied. However, future surveys of New Zealand streams during periods of variable flows, ideally focusing on catchments where pesticide applications on land are known to be high, are required to confirm this.

5. Conclusions

Improving the ecological realism of ecotoxicological studies is crucial for providing the scientific knowledge needed to regulate pesticide use more safely in the future. Our study starts to address this challenge by combining low, field-realistic concentrations of imidacloprid with two natural stressors.

Our findings suggest that periods of food shortage may worsen the impact of exposure to imidacloprid on stream mayfly populations and, ultimately, the stream and terrestrial food webs they are vital to [6]. Thus, we demonstrate the importance of integrating ecological concepts (i.e., the effects of natural stressors such as food shortages) into studies of contaminant effects on freshwater ecosystems.

Worldwide, there are an increasing number of studies assessing the toxicity of neonicotinoids to non-target organisms, but very few that explore potential interactions with natural or anthropogenic stressors that may worsen the impacts of neonicotinoids on these organisms in real ecosystems. Many more such multiple-stressor experiments are required, including experiments involving realistic stream food webs in open-system mesocosms that permit natural immigration and emigration of stream biota, in order to assess the broader effects of neonicotinoids on community structure and ecosystem processes.

It is also necessary to perform more ecological multiple-stressor field studies aimed at evaluating to which extent the current pesticide regulation may in fact be protective, for example by surveying benthic invertebrate community compositions in multiple streams spanning wide gradients of pesticide concentrations combined with other agricultural stressors. Such studies would progress real integration between the fields of freshwater ecology and ecotoxicology, a long-term goal we believe to be of utmost importance if the future biological integrity of our freshwater ecosystems is to be maintained or improved.

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