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Effects on Life-History Traits of *Hypogastrura viatica* (Collembola) Exposed to Imidacloprid Through Soil or Diet

Silje Marie Kristiansen,[a,*] Katrine Borgå,[a] Jan Thomas Rundberget,[b] and Hans Petter Leinaas[a]

[a]Department of Biosciences, University of Oslo, Oslo, Norway
[b]Norwegian Institute for Water Research, Oslo, Norway

Abstract: Collembola (springtails) are important members of soil communities worldwide by contributing to degradation of organic matter. In nature, Collembola might be exposed to the neonicotinoid insecticide imidacloprid, which is fairly persistent in soil. We exposed the widespread *Hypogastrura viatica* to imidacloprid through soil or food and monitored the animals during exposure and a post exposure period. We recorded effects on life-history traits affecting individual fitness, that is, mortality, behavioral activity, several reproduction traits, and molting frequency. Exposure through soil led to a concentration-dependent mortality, while the mortality from dietary exposure possibly reflected reduced feeding activity. The body burden of imidacloprid in the Collembola did not differ between treatments. We found no sign of recovery in behavioral activity following exposure in either experiment. The egg production of *H. viatica* was not significantly affected by imidacloprid at 0.01 mg/kg dry soil but showed a tendency to reduce number of eggs per batch and reduced hatching success. At higher concentrations, reproduction was close to, or completely, stopped. The molting frequency decreased during exposure, while in the post exposure period, we saw milder effects at the highest concentrations, suggesting elimination through molting or reduced toxic response as a result of reduced feeding activity. Overall, *H. viatica* was more sensitive to imidacloprid than previously studied Collembola, which highlights the importance of considering species sensitivities when risk-assessing soil environments. *Environ Toxicol Chem* 2021;40:3111–3122. © 2021 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Ecotoxicology; Insecticide; Invertebrate toxicology; Soil invertebrates

INTRODUCTION

Collembola (springtails) are important members of soil communities worldwide, contributing to decomposition of organic matter (see Petersen & Luxton, 1982; Rusek, 1998) and being prey to mites and spiders (Baatrup et al., 2006; Wise et al., 1999). The Collembola taxa contains approximately 8000 species (Bellinger et al., 1996–2013), which are present in almost all terrestrial habitats globally (Hopkin, 1997). Collembola display a large spectrum in life-history traits (Siepel, 1994), with varied resilience to natural stress (Malmström, 2012) and anthropogenic stress, such as pollution (Szabó et al., 2020). Like all organisms, Collembola allocates resources to different life-history traits to maximize their fitness (Stearns, 1992). Toxic substances may affect vital rates such as survival, egg production, hatching success, and juvenile growth and development (Amorim et al., 2012; Schnug, Leinaas, et al., 2014), possibly leading to long-term consequences at the population level. The neonicotinoid insecticide imidacloprid has been used worldwide to protect crops against insects (Goulson, 2013) by affecting the central nervous system and causing paralysis and death (Simon-Delso et al., 2015). More than 90% of the imidacloprid on treated crops typically enters the soil (Goulson, 2013), and its high water solubility facilitates transportation with water runoff (Bonmatin et al., 2015). Imidacloprid is banned from insecticide usage outdoors in Europe (European Commission, 2018, Regulation [EU] No. 2018/783); however, with a half-life in soil ranging between 28 and 1250 days (Goulson, 2013), it may cause long-term exposure to important soil organisms, such as Collembola.

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* Address correspondence to s.m.kristiansen@ibv.uio.no
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For Collembola, laboratory tests are standardized for studying effects on survival and recruitment from pollutant exposure (International Organization for Standardization, 1999, 2011; Organisation for Economic Co-operation and Development, 2009), mainly on the model species *Folsomia candida* (Fountain & Hopkin, 2005). Atypical for Collembola, *F. candida* is a parthenogenic species rarely found in natural soils but present in many cave systems (see Bernard et al., 2015) and in human-made habitats, being spread via flower pots and gardening soil (Hopkin, 1997). Because of its highly specialized way of life and habitat occupation, there is a need for additional ecotoxicological knowledge on other, more widespread and commonly occurring Collembola species (Son et al., 2007). This need is emphasized by differences in sensitivity found between *F. candida* and alternative species, reported for imidacloprid (de Lima e Silva et al., 2021), several metals (Nakamori et al., 2008; Santorufo et al., 2012), and an organophosphorous insecticide (Badejo & Van Straalen, 1992). Often, the nonstandardized test species was more sensitive (Greenslade & Vaughan, 2003; Son et al., 2007). Thus, effects from one or a small number of species cannot be uncritically extrapolated to a whole taxon (Schnug, Jensen, et al., 2014). In the present study, we focus on *Hypogastrura viatica*, a surface-dwelling Collembola which is widely distributed in temperate and arctic regions (Fjellberg, 1998; Jensen et al., 2006). It has sexual reproduction and is easy to culture in the laboratory, and thus suitable for ecotoxicological experiments.

The standardized toxicity tests for Collembola are designed for soil exposure because exposure to pollutants through soil porewater is considered the most important pathway for soil organisms (Van Gestel, 1997). However, in nature, they also experience dietary pollutant exposure, which is important to include to mimic the ecologically relevant conditions of Collembola. Living on the surface, it is likely that dietary exposure can play a bigger role than the ambient soil environment for *H. viatica* compared to soil-dwelling Collembola species, including *F. candida*. When exposed to contaminated food, the animals have the opportunity to avoid exposure by reducing their feeding activity (Filser & Hölscher, 1997), and terminating imidacloprid exposure can to some extent result in post exposure recovery (Azevedo-Pereira et al., 2011; Sengupta et al., 2020).

Imidacloprid exposure at sublethal concentrations reduce juvenile recruitment in Collembola (de Lima e Silva et al., 2017, 2021; van Gestel et al., 2017); however, the mechanisms involved are rarely studied (Sengupta et al., 2020). Traditional soil exposure conceals the Collembola, preventing any monitoring during exposure. Egg production, egg development time, and hatching can be assessed when the animals are kept on a surface (Campiche et al., 2006; Lee et al., 2019), such as plaster of Paris. Hatching success is highly sensitive to pollution stress (Haque et al., 2011; Lee et al., 2019), and detailed determination of such reproduction endpoints increases knowledge on the toxic mechanisms underlying recruitment.

Collembola molt their exoskeleton regularly throughout their life (Birkemoe & Leinaas, 2000), and apart from being necessary for growth, molting has an important excretion function. During molting, the lining of the midgut is excreted and renewed (Humbert, 1979), removing harmful products such as metals (see Joosse & Buker, 1979; Pawert et al., 1996; Posthuma et al., 1992). Elimination of organic contaminants through molting has not been studied, but it seems plausible that it can occur and that exposure to pollutants might induce an increased molting frequency, as seen for other arthropods (Al-Badran et al., 2019). On the other hand, exposure to some organic pollutants has been shown to disrupt and reduce the molting of Collembola (Lee et al., 2019; Zhang & Qiao, 2020), causing a reduction in excretion capacity.

The overall aim of the present study was to determine effects on life-history traits in *H. viatica* exposed to imidacloprid through soil or diet. We compared responses between exposure routes and between the exposure and post exposure periods to determine recovery. We report effects on mortality, multiple reproduction endpoints, and molting frequency from an exposure concentration range that includes those found in agricultural areas (Silva et al., 2019). In addition, we report on behavioral activity by establishing categories of visible effects for this novel species in toxicity testing and measured concentrations of imidacloprid and metabolites in Collembola. To the best of our knowledge, our study includes the first exposure experiments of any toxicant on the widespread and ecologically important Collembola *H. viatica*. By providing information on the toxic sensitivities of its life-history traits, our approach will also contribute to a more general understanding of population-level effects of Collembola.

MATERIALS AND METHODS

Test organism

Our experimental population of *H. viatica* originated from a sample of several thousand animals collected at a high Arctic site; Fjortende Julibukta in Krossfjorden, west Spitsbergen, Svalbard, Norway (79°7′N, 11°52′E). Prior to experiments, the animals were cultured for 9 months at 15 °C, equivalent to two to three generations, thereby removing parental and environmental effects. Cultured animals were kept in plastic containers (3.5 cm diameter, 3.5 cm high) with a moistened base of plaster of Paris darkened by charcoal (~0.4 g charcoal/100 g plaster). The Collembola were offered pieces of bark covered by a crust of Cyanobacteria, which is found to be a preferred food (H. P. Leinaas, personal observation). The bark was collected from *Tilia cordata* trees at the University of Oslo campus and defaunated by freezing at –80 °C for 24 h.

Experiments

We performed two experiments (Figure 1), exposing adult *H. viatica* to imidacloprid for 14 days through their ambient soil environment or through their diet. Following soil exposure, animals were given a recovery period of 28 days in a clean environment, while dietary exposure was followed by 35 days in a clean environment. The reason for this duration difference

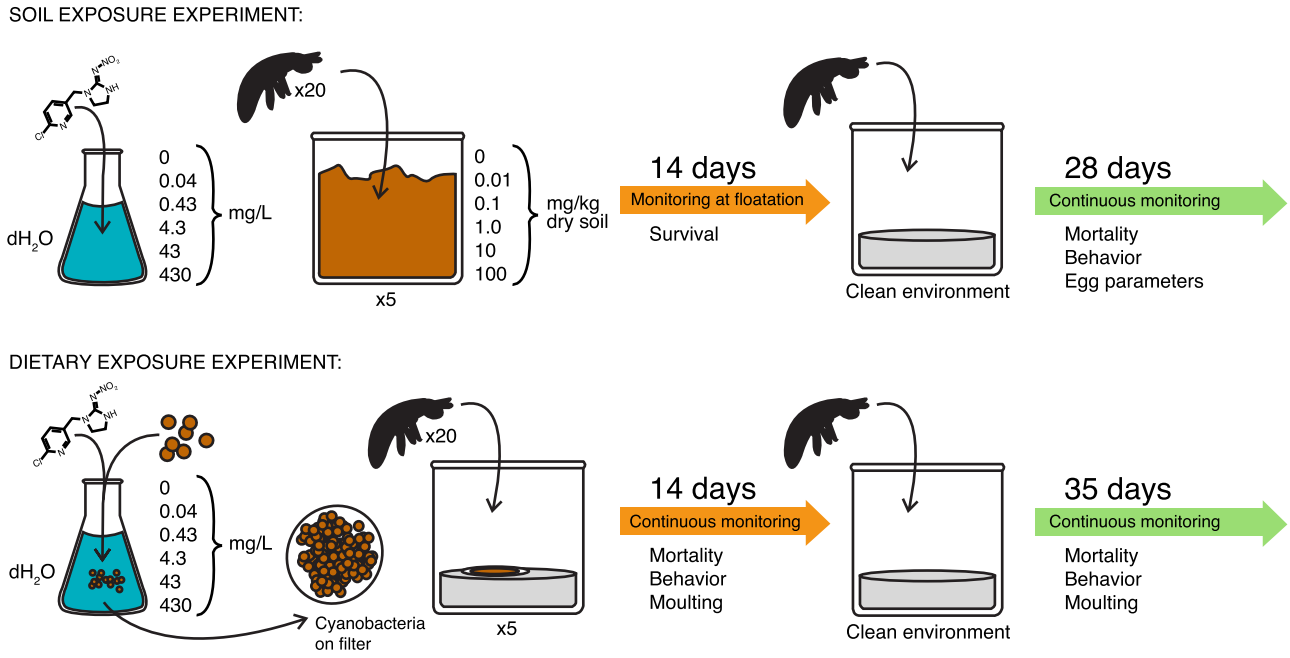

FIGURE 1: Overview of the experimental design for exposure of *Hypogastrura viatica* to imidacloprid through soil and diet (Cyanobacteria on filter).

is given below (see *Dietary exposure*). The animals were kept at 15 °C, a temperature promoting frequent reproduction (H. P. Leinaas and S. M. Kristiansen, personal observation). A continuous photoperiod imitated the midnight sun of the arctic summer, when the animals are active. All treatments and controls consisted of five replicates containing 20 adult *H. viatica*.

Soil exposure

Imidacloprid (Chemical Abstracts Service no. 138261-41-3; Sigma-Aldrich) was dissolved in distilled water and serially diluted by a factor of 10 to obtain five nominal soil concentrations in natural standard soil, LUFA 2.2 (Speyer). Together with the control, the following concentrations were obtained: 0, 0.01, 0.1, 1, 10, and 100 mg/kg dry soil. Each solution was thoroughly mixed before an amount equivalent to 4.3 g dry soil was added to each test container. Control soil was moistened with distilled water. Another 50 g soil per treatment was stored at –20 °C until chemical analysis of imidacloprid.

Approximately 20 adults (17–24 individuals) per replicate secured a satisfactory number of both sexes because sex cannot be distinguished for living *H. viatica*. We transferred randomly ten animals to each container before we added another ten, to avoid subconsciously biased sampling of the largest individuals first. During soil exposure, *H. viatica* was offered Cyanobacteria scraped off the bark and placed on the soil surface. After 14 days, the animals were carefully collected by flotation, and those not found were assumed dead. Collembola were transferred to new, clean containers with a base of plaster and charcoal and presented with pieces of bark with Cyanobacteria, which was replaced every 3 days to avoid fungi growth. The post exposure period lasted 28 days, and the animals were discarded at the termination of the experiment.

Dietary exposure

The Cyanobacteria crust was scraped off slices of bark and homogenized to a fine powder. Imidacloprid was dissolved in distilled water, obtaining nominal concentrations of 0, 0.04, 0.22, 0.43, 2.2, and 4.3 mg/L in the solution. These solutions would result in concentrations of 0, 0.01, 0.05, 0.1, 0.5, and 1 mg/kg dry soil had these solutions been mixed in soil in a similar manner as for the soil experiment. There were no studies applying dietary exposure at the time of the experiment, and we therefore chose solution concentrations that were in the same range as the solutions used in the soil experiment. Six bulks of homogenized Cyanobacteria were soaked in the imidacloprid solutions in the dark at 4 °C overnight, to ensure equilibrium (control food was soaked in distilled water), before being filtered onto cellulose nitrate membrane filters (pore size = 0.45 µm, diameter = 25 mm; Sartorius) and dried in the dark at room temperature. The prepared filters were divided into four pieces and stored in the dark and dry until feeding. The limited Cyanobacteria material did not allow for chemical analysis, and it is unclear how much imidacloprid the Cyanobacteria soak up. However, the identical method was applied in Sengupta et al. (2020), where three of five concentrations were similar, including the lowest and highest concentrations. Their concentration range was between 0.2 and 290 mg/kg bark, and we can thus assume that our Cyanobacteria treated with imidacloprid were within the same range. The analyzed concentrations in Sengupta et al. (2020) confirm an increasing concentration range in spiked Cyanobacteria in accordance with increasing concentration in the soaking solution.

Each test container with a moistened base of plaster with charcoal received 18 to 20 adult *H. viatica* and with a one-fourth filter with Cyanobacteria. Filters were replaced every 3 days, ensuring ad libitum food. After 14 days of exposure, the Collembola were transferred to clean test containers and given nonspiked food for 35 days, that is, 1 week longer than for the soil experiment. The animals were stored at –20 °C until chemical analysis of internal concentrations.

We prolonged the post exposure period because control animals stopped reproducing 3 days after the exposure stopped, which indicated that their living conditions were not optimal. After 14 days of no reproduction by the controls, we changed foraging procedure for all treatments and replaced the filters with pieces of bark with Cyanobacteria. Egg production resumed in the control after 6 days (day 37 of the experiment), and the experiment was terminated on day 49. The halted reproduction in the post exposure period removed our basis for drawing any conclusions concerning any reproduction parameters. Therefore, data on reproduction from the diet experiment were not analyzed or interpreted in the present study. All experimental boxes for controls and treatments were treated similarly and were never without food. We consider this experiment to contribute valuable knowledge on other endpoints besides reproduction and present benefits with such an alternative approach to soil exposure.

Categories of behavioral activity

To assess visible effects of imidacloprid, individual *H. viatica* were observed every third day and grouped into five behavioral categories: 1) *unaffected*, actively walking, jumping, and foraging; 2) *mildly affected*, low activity level in walking, no jumping, and often with condensation droplets on the body, owing to reduced activity; 3) *visible spasms*, staggering walk and spasms, lifting of the hind part of the abdomen, and abnormal antennae movements; 4) *moribund*, heavy spasms and immobilization, lying on the side; 5) *dead*, stiff body, no sign of life.

Behavioral activity was assessed throughout the diet experiment but only during the post exposure period of the soil experiment because the animals could not be observed while in the soil. The categories were defined early in the experiment and documented with videos and photographs (example imagery in Supporting Information, Figures S1–S5). When analyzing lethal toxicity, we lumped the categories *moribund* and *dead* into one group, that is, *functional mortality*, because the moribund individuals were not observed to recover. Animals that died during the experiments were discarded. The categorization system for *F. candida* (Houx et al., 1996) does not fit the behavior of *H. viatica*.

Reproduction parameters and molting

We recorded egg production, hatching success, egg development time, and juvenile size. Egg production was measured by counting the number and sizes of egg batches laid since last inspection. The day-fresh eggs were determined by light yellow color and transferred to separate containers to daily monitor hatching. At the day of hatching, the new juveniles were harvested in 70% ethanol and heated to approximately 70 °C to stretch their bodies. Juvenile sizes were analyzed by photographing them under a stereomicroscope and measuring their lengths with Leica Application Suite 4.5 software. Reproduction parameters were recorded for both experiments but not analyzed for the diet experiment because of the halted egg production in the control.

In the diet experiment, we counted the number of molted exoskeletons at each inspection before discarding them. Molting was not registered in the soil experiment because the animals were hidden in the soil during the exposure period.

Chemical analysis of Collembola

Replicates of Collembola from one treatment of the dietary exposure experiment were pooled (5–19 individuals) and analyzed for internal concentrations of imidacloprid and metabolites at the Norwegian Institute for Water Research (NIVA) in Oslo. The metabolites included imidacloprid-olefin, 5-hydroxy-imidacloprid, desnitro-imidacloprid, imidacloprid-urea, desnitro-olefin-imidacloprid, and 6-chloronicotinic acid. Samples were extracted with 0.1 ml acetonitrile before being sonicated and centrifuged repeatedly ten times. The acetonitrile extract was transferred to a new vial, evaporated to dryness under nitrogen, and resolved in 0.05 ml of 10% acetonitrile in water prior to ultraperformance liquid chromatographic (UPLC)–mass spectroscopic (MS) analysis. The UPLC-MS method was as described elsewhere (Sengupta et al., 2020) with minor adjustments. Screening of imidacloprid and its metabolites was performed with multiple reaction monitoring in positive ionization mode; imidacloprid 256 > 175, 256 > 209; imidacloprid-olefin 254 > 171, 254 > 205; 5-hydroxy-imidacloprid 272 > 191, 272 > 225; desnitro-imidacloprid 211 > 90, 211 > 126; imidacloprid-urea 212 > 78, 212 > 128; desnitro-olefin-imidacloprid 209 > 90, 209 > 126; and 6-chloronicotinic acid 158 > 78, 158 > 122. The limits of detection (LODs) were estimated to be 0.2 ng/sample for 6-chloronicotinic acid and 0.02 ng/sample for the other analytes. Collembola exposed to relatively lower imidacloprid concentrations through soil (where mortality was lower) were pooled and applied as a pilot test for chemical analysis.

Quality assurance of soil

Experimental soil was analyzed for imidacloprid at NIVA, as described by Sengupta et al. (2020). The LOD was 0.0001 mg/kg dry soil. We analyzed one soil sample from each treatment and five subsamples of the nominal concentration 0.1 mg/kg dry soil. Prior to imidacloprid analysis, frozen soil was freeze-dried in a Leybold-Heraeus GT2 Freeze dryer with a Leybold Vakuum vacuum pump (Leybold).

The analysis of soil with nominal imidacloprid concentrations of 0, 0.01, 0.1, 1, 10, and 100 mg/kg dry soil measured 0, 0.01, 0.09, 0.74, 8.2, and 73 mg/kg dry soil, respectively. The mean imidacloprid concentration of the subsamples with the nominal concentration of 0.1 mg/kg dry soil was 0.097 mg/kg dry soil (±0.005 standard deviation, $n = 5$), confirming a sufficiently

homogenous distribution of imidacloprid in the soil. We were unable to analyze the spiked bark used in the diet experiment because of limited material.

Data treatment and statistical analysis

All statistical analyses were conducted using Rstudio, Ver 3.5.1. (R Foundation for Statistical Computing, 2018), with a 5% significance level. Functional mortality (moribund + dead) from the soil experiment was analyzed by fitting concentration–response models to the experimental data using the add-on package "drc: Dose-Response Analysis" (Ritz et al., 2015). Models were fitted both to experimental data applying the nominal concentrations and to the data using the measured concentrations because two of the concentrations had a deviance exceeding 20%. A model selection was carried out based on Akaike's information criterion, estimating residual variance and a lack-of-fit test. For the proportion of animals being functionally dead at the end of exposure and the end of post exposure, a four-parameter and a two-parameter log-logistic model had the best fit, respectively, which was the case for both nominal and measured concentrations. The fitted models were used to calculate the concentration required to be lethal to 10% and 50% of tests animals (LC10 and LC50, respectively).

Functional mortality in the diet experiment did not meet the requirements of drc models, and concentration descriptors (LC*x*) could thus not be reliably calculated because of low mortality. We instead estimated Kaplan-Meier survival functions using the "survival" package (Therneau, 2020) and tested differences between treatments with a log-rank test. The few Collembola that appeared to be falsely categorized as moribund, apparent from the following checkup having visible spasms, were not included as functionally dead.

Internal body concentrations of imidacloprid were standardized to the number of *H. viatica* (5–19 individuals) because of low sample weight. Internal concentrations between imidacloprid treatments (excluding control) were tested with a Kruskal-Wallis rank sum test. Imidacloprid-olefin concentrations were not tested because of the high number of concentrations below the LOD.

Reproduction parameters were only analyzed for the soil experiment because of halted egg production in the control of the diet experiment. Because sexes could not be distinguished, egg production was expressed as the cumulative number of eggs produced per animal, rather than per female. Difference in egg production between control and 0.01 mg/kg dry soil was tested with the Wilcoxon signed-rank test. Egg production was not sufficient at higher concentrations for statistical testing; only one batch of eight eggs was produced at 0.1 mg/kg dry soil. A two-proportions *z* test was used to test for difference in proportions of eggs hatched, that is, hatching success. To assess the relationship between batch size and hatching success, we fitted a generalized linear mixed effect model (glmm), using the R package "lme4" (Bates et al., 2015). Single eggs were treated as replicates with a binomial distribution (hatched/not hatched), accounting for random effect by eggs being from the same batch. The single batch of eight eggs from 0.1 mg/kg dry soil was included in the glmm. The best-fit model, found using a deviance test, included fixed effects of *imidacloprid concentration* and *batch size*, the latter defined as a categorical variable with three levels: *small* (1–4 eggs), *medium* (5–9 eggs), and *large* (>10 eggs). Egg development time was measured as number of days, and single eggs were treated as replicates. The difference in egg development time between treatments was tested using the Wilcoxon signed-rank test. For juvenile size, single Collembola were treated as replicates, and a two-sample Student *t* test was used to assess differences between treatments.

The cumulative number of molts per animal from the diet experiment was estimated from the number of exuviae in each replicate divided by the number of animals alive per time interval. A two-parameter log-logistic model (drc) was fitted for the exposure period and a third-order polynomial regression model for the post exposure period. Polynomial order was selected using an *F* test.

RESULTS

Functional mortality

The mortality of control animals was <20% in both experiments, which is a validity criterion in other standard procedures (Organisation for Economic Co-operation and Development, 2009). In the soil exposure experiment, functional mortality of *H. viatica* increased with imidacloprid concentration for both the exposure period and the post exposure period (Figure 2). Both periods resulted in a sigmoid response curve, with no clear effect at 0.01 mg/kg dry soil, followed by a steep increase until 1.0 mg/kg where only few or no animals survived. At the end of the post exposure period, all animals at the three highest concentrations were dead. The LC10 and LC50 values were comparable between the exposure and post exposure periods, with overlapping confidence intervals (Figure 2). The LC50 estimate for the exposure period was 0.15 mg/kg dry soil (95% confidence interval 0.11–0.19) when the model was fit to measured imidacloprid concentrations. The other LC*x* values were identical to those calculated from nominal concentrations.

With dietary exposure, survival was negatively affected by concentration during both the exposure (log-rank test: $\chi^2 = 80.6$, $df = 5$, $p = «0.01$) and the post exposure (log-rank test: $\chi^2 = 25.6$, $df = 5$, $p < 0.05$) periods. However, during the post exposure period, even the lowest concentration resulted in >40% functional mortality, while only moderate additional mortality was seen at the higher concentrations (Figure 3). The high exposure concentration of food soaked in 2.15 mg/L imidacloprid solution caused lower mortality than the lower solution concentration of 0.43 mg/L. The LC10 and LC50 values could not be reliably calculated because of the low mortality.

Body burden

Imidacloprid was quantified in all *H. viatica* samples from the diet experiment, whereas there was no detectable residue in

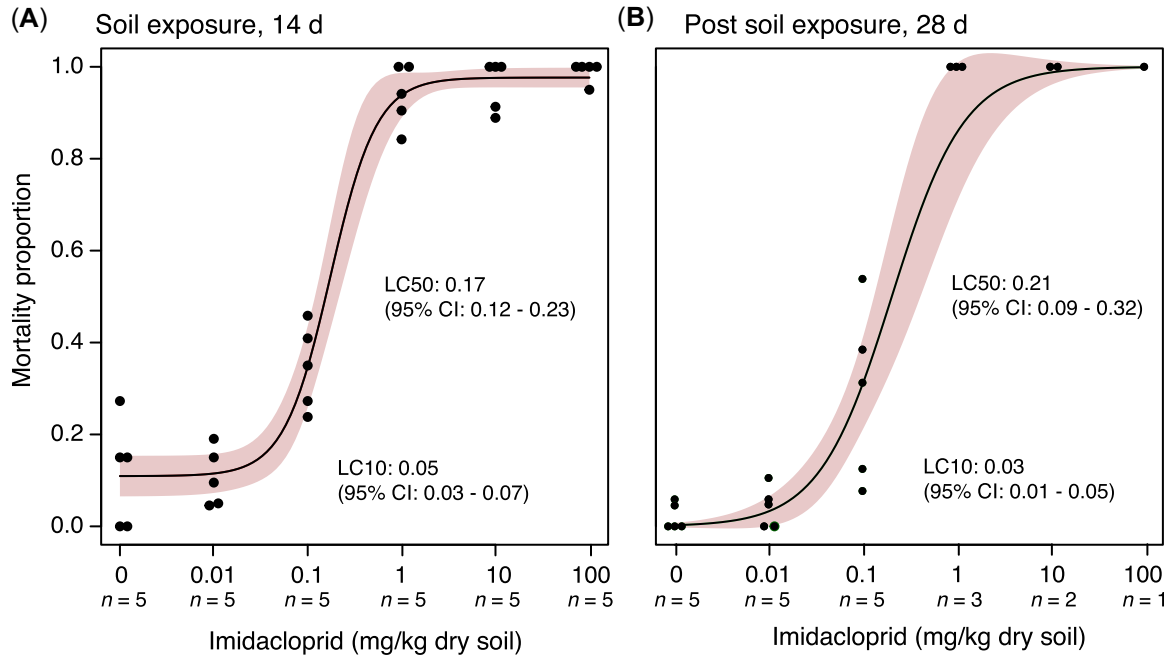

FIGURE 2: Proportion of functional mortality (dead + moribund) of adult *Hypogastrura viatica* exposed to imidacloprid in soil (**A**), followed by a post exposure period (**B**). Curves are fitted according to a four-parametric log-logistic curve (**A**) or a two-parametric log-logistic curve (**B**). In (**B**), the total number of animals per treatment comprised those that survived the exposure period of 14 days. The colored area represents the 95% confidence interval. Lethal concentrations (milligrams per kilogram dry soil) for 10 and 50% (LC10 and LC50) of the test organisms were calculated from the fitted models and are presented with their 95% confidence intervals.

the controls (Table 1). The internal concentrations did not differ between treatments (Kruskal-Wallis rank sum test, $\chi^2 = 6.26$, $df = 4$, $p = 0.18$), and thus did not reflect the increasing concentration in the food. Imidacloprid-olefin was the only metabolite detected, and it was present in all treatments except at the intermediate solution concentration 0.43 mg/L.

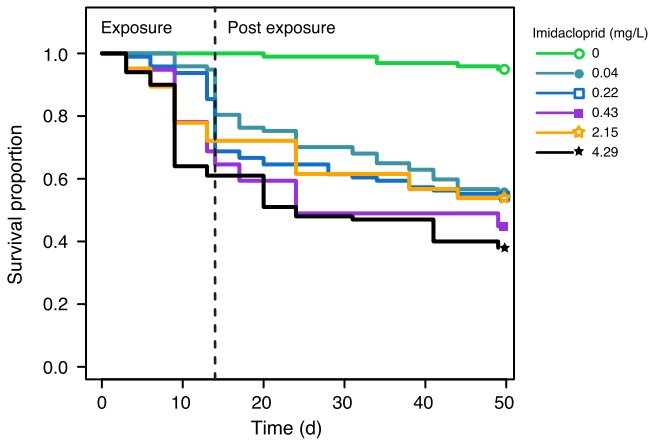

FIGURE 3: Kaplan-Meier survival estimates of adult *Hypogastrura viatica* exposed to imidacloprid through diet. Each treatment included five replicates of approximately 20 animals. Results were obtained from 14 days of exposure, followed by a post exposure period of 35 days, separated by the vertical dashed line. Survival comprised animals that were neither moribund nor dead. The food consisted of bark spiked with solutions of imidacloprid (milligrams per liter).

Behavioral activity over time

In the post exposure period of the soil experiment, the lowest concentration of 0.01 mg/kg dry soil gave no indication of delayed toxicity during the post exposure period (Figure 4A). At 0.1 mg/kg dry soil, unaffected animals became mildly affected, those with visible spasms became moribund, and the moribund animals died with time. The three highest concentrations caused functional mortality (moribund + dead) in nearly all animals after exposure at day 14 (Figure 4A), and a high proportion of moribund individuals died with time.

In the diet experiment, only a few animals died during the exposure period, but the animals' condition worsened with time in all treatments (Figure 4B). This was particularly evident for the number of moribund individuals. During the post exposure period, the steady increase of dead animals reflected the dying of moribund animals because the proportion of functionally dead appeared close to constant over time (Figure 4C). The proportion of visible spasms and the grouping of the two mildest categories (unaffected and mildly affected) also appeared constant over time. Thus, there were no indications of recovery. The proportions of behavioral activity appeared similar at the three highest concentrations.

Reproduction

In the soil experiment, the lowest imidacloprid concentration did not significantly affect egg production (Figure 5A; Wilcoxon test: $W = 8.5$, $p = 0.45$), but the egg-laying pattern

TABLE 1: Internal concentrations of imidacloprid[a]

Nominal exposure solution (mg/L)	Imidacloprid (ng/individual wet wt)				Imidacloprid-olefin (ng/individual wet wt)			
	Mean[b]	Minimum–maximum	SD	*n*	Mean[c]	Minimum–maximum	SD	*n*
0	<LOD	<LOD	na	5	<LOD	<LOD	na	5
0.04	0.13	0.09–0.17	0.04	5	0.002	<LOD–0.004	0.001	5
0.22	0.09	0.04–0.10	0.03	5	0.001	<LOD–0.003	0.002	5
0.43	0.14	0.11–0.18	0.03	4	<LOD	<LOD	na	4
2.2	0.12	0.11–0.13	0.01	5	0.002	<LOD–0.006	0.003	5
4.3	0.11	0.09–0.13	0.02	5	0.003	<LOD–0.012	0.005	5

[a]Internal concentration of imidacloprid and imidacloprid-olefin in adult *Hypogastrura viatica* (Collembola) following 14 days of dietary exposure and 35 days of recovery. Because of low sample material, the concentrations were standardized to the number of animals. The food, Cyanobacteria scraped off bark, was soaked overnight in solutions of imidacloprid (milligrams per liter).
[b]No significant differences among treatments ($p = 0.18$).
[c]Differences between treatments were not tested because of the high LOD. When calculating the mean of imidacloprid-olefin, <LOD was set to 0. The pooled sample concentration was divided by the number of animals, explaining values below LOD.
SD = standard deviation; LOD = limit of detection (0.02 ng/individual wet wt); na = not available.

differed, with 41% small batches (<5 eggs), while no small batches were laid by control animals. No reproduction occurred at any higher concentrations, except for one batch of eight eggs laid at 0.1 mg/kg dry soil. This single reproductive event was insufficient for a reliable test and therefore excluded from the remaining reproduction parameters (Figure 5B–D). Imidacloprid reduced hatching success with 7 to 24% (Figure 5B; 2 proportions z test: $\chi^2 = 11.2$, $p = 0.0008$) and was related to small batches (glmm: $z = -2.932$, $p = 0.003$). The egg development time was prolonged and had higher variation with imidacloprid (Figure 5C; Wilcoxon test: $W = 2657.5$, $p < 0.001$). Lastly, imidacloprid increased the body sizes of juveniles (Figure 5D; t test: $t = -2.98$, $p = 0.003$). Egg parameters were not analyzed from the dietary exposure experiment because of limited reproduction.

Molting frequency

During dietary exposure, the cumulative number of molts declined with increasing imidacloprid concentration in the soaking solution (Figure 6A; log-logistic model: $t = 2.19$, $p = 0.037$). Following exposure, the molting frequency declined at the lowest concentrations, but it relatively increased at the two highest (Figure 6B; polygonial model: $F = 3.7$ on 3 and 26 *df*, $p = 0.024$).

DISCUSSION

Functional mortality and the effect of exposure route

The functional mortality in the two experiments revealed that soil exposure was clearly concentration-dependent, while dietary exposure reflected a more complex causality. The soil experiment confirmed that soil and porewater are important exposure routes for Collembola (Ogungbemi & van Gestel, 2018; Styrishave et al., 2010) and for this surface-dwelling species. The functional mortality during the post exposure period reflected a long-term toxic effect of imidacloprid, with no indication of recovery, possibly due to the lasting binding of imidacloprid to the receptors (Matsuda & Sattelle, 2004). The dietary exposure also showed concentration-dependent functional mortality in the lower nominal concentration range, but in the higher range, cumulative mortality had a relatively low increase, possibly because of reduced feeding activity. At the three highest concentrations, most animals were inactive and experienced spasms, and their defective health likely reduced their normal behavior, including their feeding activity. Reduced feeding was further indicated by observation of intact Cyanobacteria on filters at the high concentrations (Supporting Information, Figure S6) compared to reduced amount of Cyanobacteria on filters with control or low concentrations (Supporting Information, Figure S1). We also observed fungal hyphae bloom on filters at high concentrations (Supporting Information, Figure S6), which would otherwise be removed by Collembola grazing (H. P. Leinaas and S. M. Kristiansen, personal observation). Reduced feeding activity could also be due to *H. viatica* actively avoiding food spiked with high concentrations to reduce exposure because imidacloprid disrupts feeding behavior in Coleoptera (Wumuerhan et al., 2020) and Hemiptera (Miranda et al., 2016). Such a behavioral effect is seen in other Collembola presented with metal-contaminated food (Filser et al., 2000). In addition, Collembola can avoid metal contamination in their soil environment (see Boiteau et al., 2011).

The internal concentrations of imidacloprid in *H. viatica* may similarly partly be explained by a low feeding rate, at least for the lack of increase at the highest exposure concentrations. Our findings of the metabolite olefin show that Collembola can biotransform imidacloprid, but we were unable to link the biotransformation capacity to their mortality because the mortality response differs between the exposure routes. If increased biotransformation reduced their mortality, it should have been reflected in both experiments. Our findings emphasize the need for more knowledge on the uptake routes of pollutants, to increase our understanding of pollutant effects on soil communities.

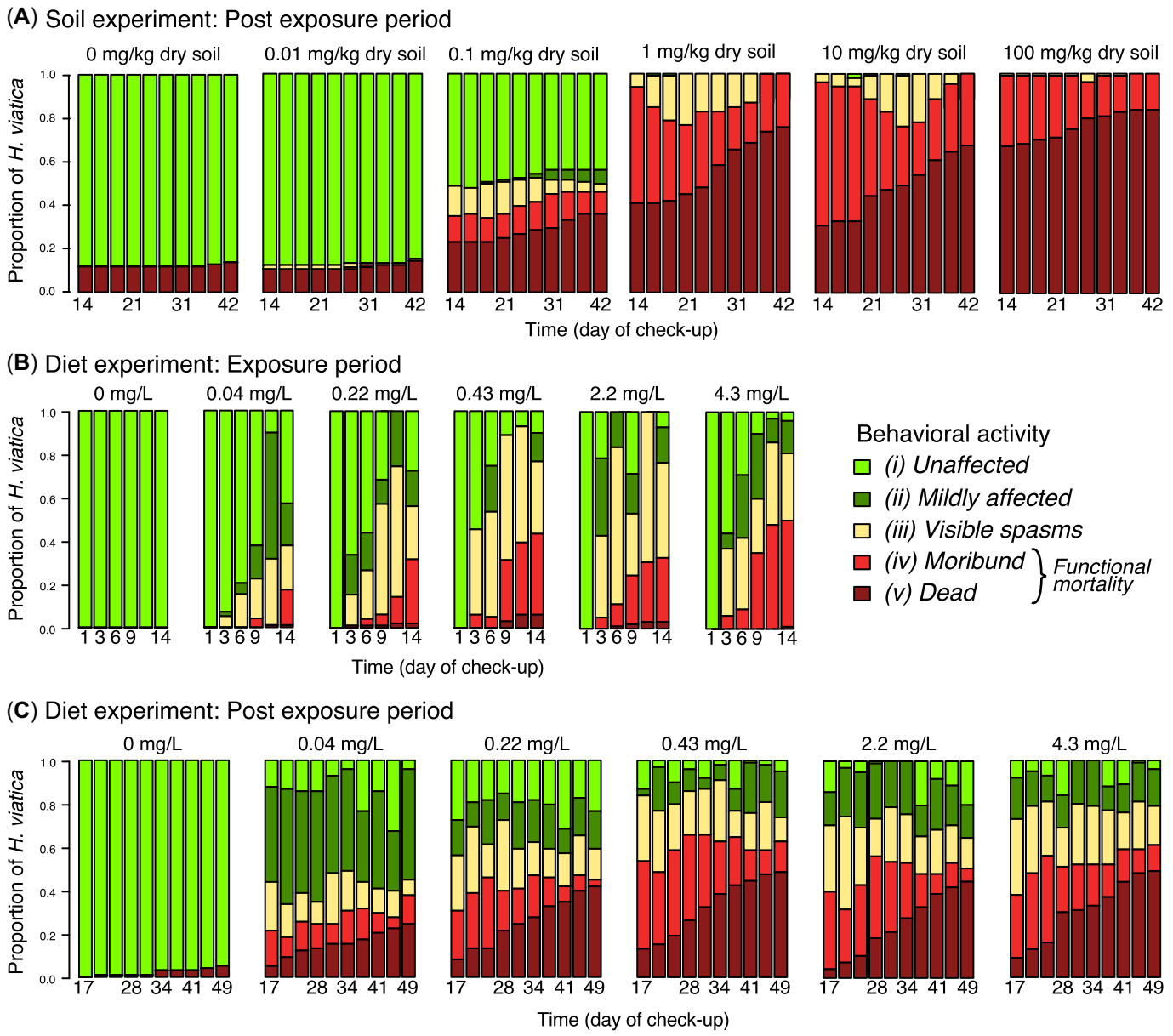

FIGURE 4: Proportion of *Hypogastrura viatica* categorized with a behavioral toxic effect caused by imidacloprid following 14 days of soil exposure (**A**), during dietary exposure (**B**), and following dietary exposure (**C**). For the soil experiment, each bar contains the total number of animals from five replicates surviving the exposure period at one time point; thus, the number of animals per bar and concentration varies. The diet experiment was monitored from the first day, and each bar consists of the total number of animals for five replicates holding 20 animals each.

Species sensitivity to imidacloprid

Hypogastrura viatica was more sensitive to imidacloprid compared to reported results on *F. candida* exposed through soil: LC50 values for *F. candida* range between 0.44 and 0.84 mg/kg dry soil (de Lima e Silva et al., 2017; Mabubu et al., 2017; van Gestel et al., 2017) and were thus at least twice the LC50 for *H. viatica* of 0.17 to 0.21 mg/kg dry soil. These studies exposed juvenile *F. candida*, while we exposed adults, which are expected to be less sensitive. Moreover, the LC50 of imidacloprid for other adult Collembola species was up to 2 orders of magnitude higher than that of *H. viatica* (0.55–39 mg/kg dry soil; de Lima e Silva et al., 2021). Compared to *F. candida*, the sensitivity for sublethal effects by imidacloprid was also markedly higher for *H. viatica*: at 0.1 mg/kg dry soil, *H. viatica* had close to complete inhibition of egg production, while *F. candida* reproduced as normal at the same concentration, defined as the no-observed-effect concentration for recruitment (de Lima e Silva et al., 2017; van Gestel et al., 2017). Sensitivity differences are also apparent for dietary exposure: when exposed to the same diet regime as our study, the adult Collembola *Folsomia quadrioculata* had at least 93% survival at all concentrations (Sengupta et al., 2020), that is, less sensitive to imidacloprid than *H. viatica*. Differences in lethal and sublethal effects from the same exposure conditions demonstrate the importance of taking species sensitivities into consideration when conducting risk assessments for soil environments. Broadening the use of

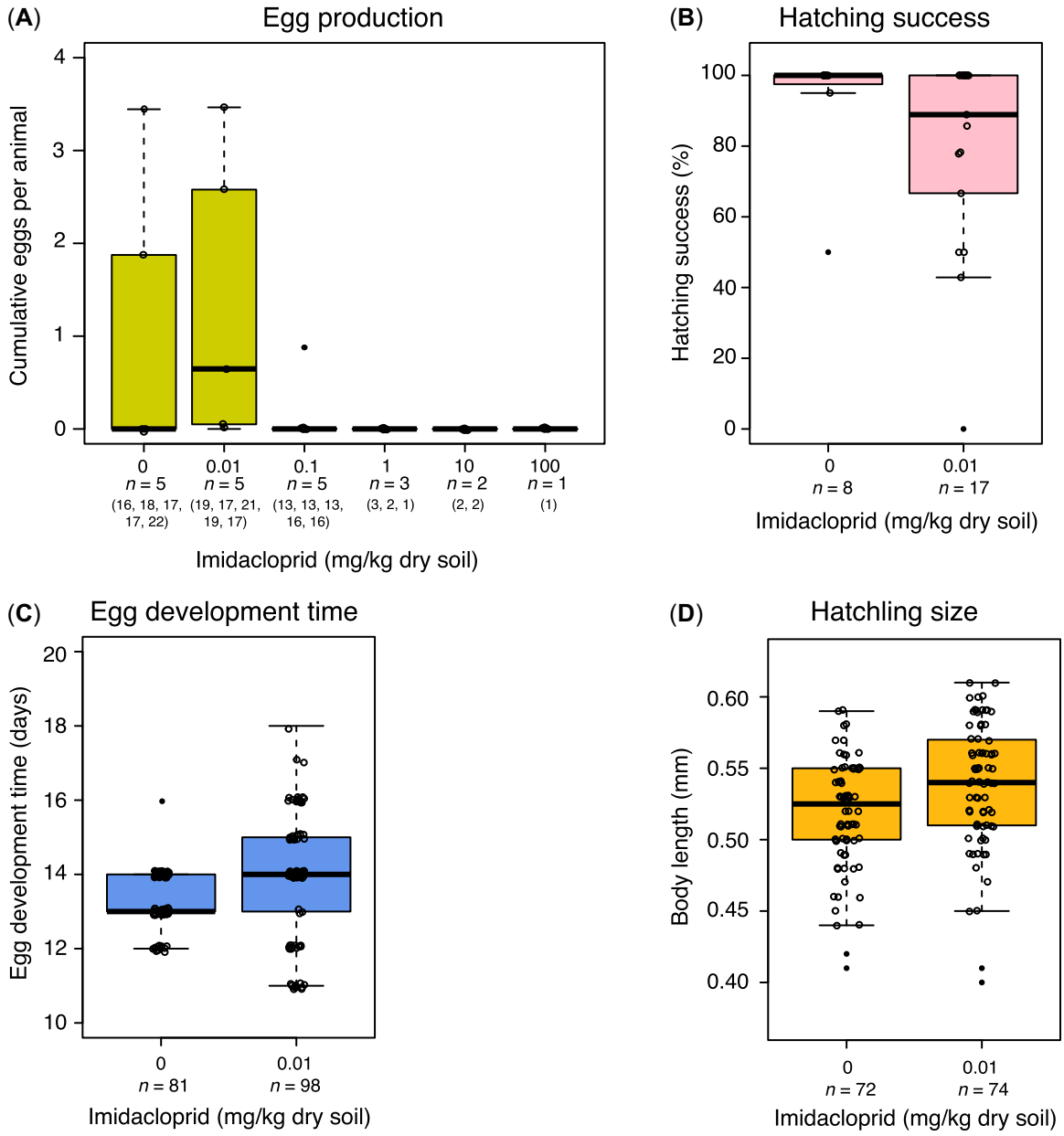

FIGURE 5: Reproductive responses of *Hypogastrura viatica* during a 28-day post exposure period, following 14 days of exposure to imidacloprid through soil. The sample size of egg production is the number of containers with living animals at day 14 (end of exposure) with the number of animals per container. The sample size for hatching success is the number of egg batches (clutch of multiple eggs laid by one individual), the sample size for egg development is number of eggs, and for juvenile size, the sample size is number of Collembola. Outliers are shown as filled circles.

Collembola species has also been suggested (see Greenslade & Vaughan, 2003; Son et al., 2007).

Potential recovery

In the post exposure period of both experiments, continued mortality and the intensified effect on behavioral activity with time gave no indication of recovery in *H. viatica* after the end of exposure. By contrast, partial recovery was found for reproduction in *F. quadrioculata* exposed to imidacloprid through diet (Sengupta et al., 2020). Recovery was also found in aquatic invertebrates (Azevedo-Pereira et al., 2011; Stoughton et al., 2008) but after pulsed exposure to imidacloprid, which is not comparable to our continuous 14-day exposure.

Effect on recruitment

Soil exposure to imidacloprid clearly reduced *H. viatica*'s fecundity (measured as number of eggs produced) because 0.1 mg/kg soil resulted in high survival but close to a complete stop in reproduction. The lowest concentration did not affect fecundity, coinciding with nearly all individuals categorized as unaffected for behavioral activity. In agreement with our findings, the sensitivity of the Collembola *Yuukianura szeptyckii* to teflubenzaron was less evident for fecundity compared to other recruitment traits, such as hatching success (Lee et al., 2019).

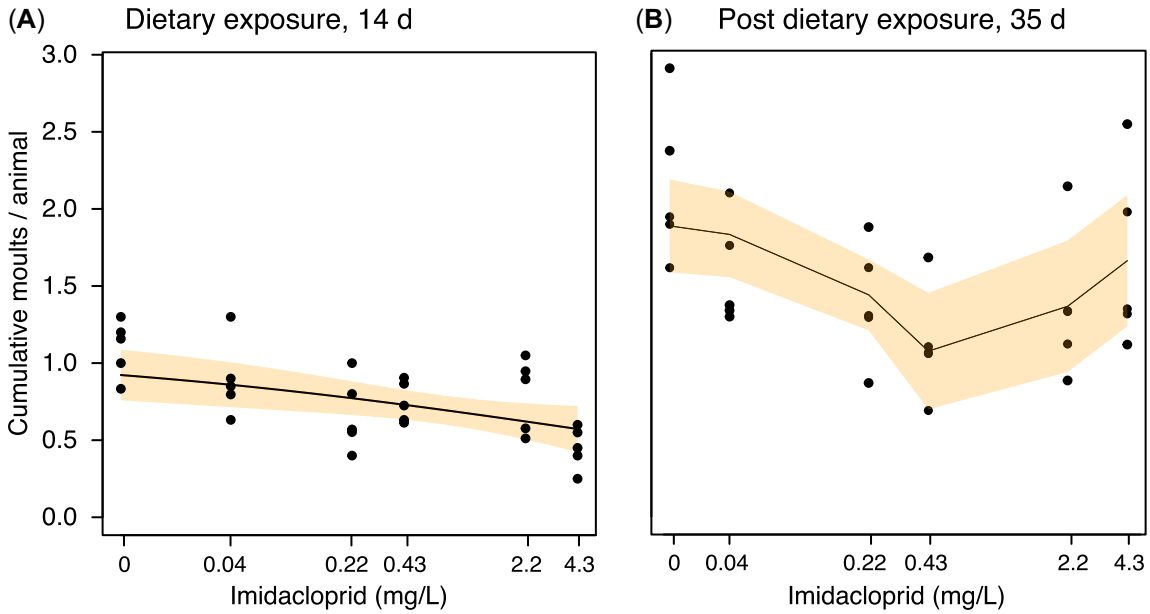

FIGURE 6: The cumulative number of molted exoskeletons per individual *Hypogastrura viatica* exposed to imidacloprid through diet (**A**), followed by a post exposure period (**B**). Curves were fitted according to a two-parametric log-logistic model (**A**) and a polynomial regression model (**B**). The colored area represents the 95% confidence interval. The food was bark spiked with solutions of imidacloprid (milligrams per liter).

However, we found a change in the egg-laying pattern, indicating a toxic response splitting egg deposition into multiple small batches. A similar stress response is seen by heat-stressed *H. viatica* at 25 °C (H. P. Leinaas and S. M. Kristiansen, personal observation). Imidacloprid reduced hatching success, in agreement with other contaminants (Haque et al., 2011; Sverdrup et al., 2001; Xu et al., 2009), which means a subsequent negative effect on recruitment. Reduced recruitment is also found for *F. candida* exposed to sublethal concentrations of imidacloprid (see de Lima e Silva et al., 2017; van Gestel et al., 2017); however, the trait determining the effect was not identified. We found that smaller egg batches were less likely to hatch, indicating a toxic response early in egg production because smaller batches were common only in animals exposed to imidacloprid. This might be the first sign of toxic stress related to recruitment, underlining the importance of including several traits affecting reproductive success.

Overall, our results indicate a toxic response in offspring quality and survival. Imidacloprid prolonged egg development time, causing the eggs to be exposed longer. However, little is known about how toxic stress of an adult Collembola affects egg development time (Smit et al., 2004). Imidacloprid resulted in larger juvenile size, which indicates that eggs were larger when laid, and thus required longer development time, possibly a response to adverse conditions (Tully & Ferrière, 2008). The sum of these reproductive parameters might affect the recruitment of *H. viatica*, and thus cause population-level effects.

Effect on molting

The reduced molting frequency during dietary exposure gave no indication of activation or up-regulation of molting to eliminate imidacloprid. Shedding the exoskeleton is highly energy-demanding and often coincides with mortality in cultured animals (H. P. Leinaas and S. M. Kristiansen, personal observation). Thus, our results might suggest that toxic stress reduces the energy allocated for molting. Contrastingly, molting remained unaffected or even increased when exposed to sublethal concentrations of herbicides (Badejo & Van Straalen, 1992; Haque et al., 2011). However, because imidacloprid targets the nervous system, it is expected to have a different effect from that of herbicides. *Hypogastrura* species synchronize their molting by chemical communication (Leinaas, 1983), and a potential disruption by pollutants in communication should be studied further.

During the post exposure period, the molting frequency declined in the lower concentration range but increased at the two highest concentrations. The latter relative increase in molting might reflect a tendency to eliminate contaminants at high concentrations by evacuation of the midgut. Similarly, an aquatic arthropod responded to imidacloprid with increased molting (Al-Badran et al., 2019). If the increased molting reflects increased elimination as seen for metals (Posthuma et al., 1992), it could be linked to both the flat mortality pattern across treatments and the similar internal concentration of imidacloprid measured in the animals. A future study with chemical analysis of the molted exoskeletons could reveal whether elimination through molting is limited to metals.

CONCLUSIONS

By being more sensitive to imidacloprid than other Collembola species tested, *H. viatica* highlights the importance of considering species sensitivities when risk-assessing soil environments. The exposure routes revealed different mortality

responses, with soil exposure being clearly concentration-dependent, while dietary exposure was likely impacted by feeding activity. By combining several reproduction parameters, we provide insight into the complex mechanisms of toxic stress on population-level effects, such as recruitment. Lastly, the molting frequency of Collembola was impaired during exposure but coincided unexpectedly with low mortality at higher treatment concentrations during post exposure, suggesting elimination.

Supporting Information—The Supporting Information are available on the Wiley Online Library at https://doi.org/10.1002/etc.5187.

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Author Contributions Statement—S. M. Kristiansen, K. Borgå, and H. P. Leinaas: experimental design and interpretation of data. S. M. Kristiansen: data acquisition through 2 laboratory experiments and data analyses and manuscript-writing. J. T. Rundberget: chemical analyses of imidacloprid compounds in soil and Collembola. All authors have revised the manuscript critically and contributed to the scientific content.

Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (s.m.kristiansen@ibv.uio.no).

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