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Dietary exposure to imidacloprid, and sub-lethal effects in springtails

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Mestrado em Biologia Humana e Ambiente

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2021

Acknowledgements

This research was part of MULTICLIM project, conducted at the Department of Biosciences at the University of Oslo (UiO) and was financed by the Research Council of Norway. It was carried out under the supervision of my main supervisor Maria Teresa Rebelo (FCUL) and Katrine Borgå (UiO), Siljie Marie Kristiansen (UiO).

First, I want to thank Silje for all the help and feedback, for all the tips, suggestions, and most of all for the support and incentive during the whole process. Thank you Silje for being the first person to approach, to understand and to explain everything from day one. Thank you for pushing me when I didn't want to write and to advise me and try to know me. Thank you Katrine Borgå for the thorough feedback and specially for always creating meetings and dinners to support and connect with us. Thanks to Sagnik for all the help in the lab with the springtails, for always being available to give some feedback or tips and for the lab talks. I had so much fun in Norway due to these people and the Tox group and learn so much about springtails and about the city itself. The meetings and the games were very funny and Drøbak was a weekend that I will never forget. I want to thank to Tox group for the support and for all the feedback.

I would like to thank Dominika for being there during Norwegian classes, and for supporting me and understanding my pain and for the shopping.

I want to thank my family that allowed me to live with them during this journey. They helped me when I needed, supported me, took care of me and most of all they were there when I just needed to talk in Portuguese instead of Norwegian or English, and listen to me.

Eduardo thank you for always being there for me, for even when we were in different countries, in a different time zone for calling me, for listening for hours, for all laughs and for not allowing me to miss home. For reassuring me and specially for taking me out of the house to get coffee when I needed and when I was impossible, you always knew what to do. It is impossible to thank you for everything that you have done for me because, first I don't remember all those things (yes, they were a lot!) and second because I know you can't remember them too.

Thank you, Carolina, and Ana, for the virtual coffee, tea, and tears and for always putting up with my mood swings and always being available to help. A special thank you to both for going with me, before it all went crazy with Covid, to the library and got me out of the house just to force me to write my thesis and because they knew that libraries and friends are my special place to write.

I would like to thank to Ana M. for supporting me and for the help and the long years that we spend talking and trying to just help each other during this crazy life. It's easier to go through college when you have someone like you nearby, and you were the first person that I met in college. You are going through the same, so you understood and so thank you for the thousand messages that we send each other with just images that mean the world.

An enormous thank you to my family, for not disowning me and putting up with all those bad days, and all those meltdowns, and specially all those days when I just needed to vent, and they all made me food.

Thank you, mom, and dad, for being there, for listening, for trying to help, but most of all for being who you are. There were a lot of times that I needed you and you were there, even when I was in Norway, you were there. You listened, you fed me, you supported me, you paid everything and made

my dreams come true for all these years, even when it meant for you to have less. I will spend my entire life thanking you and trying too just be someone you would be proud of.

Thank you to Maria Teresa Rebelo for accepting to be my supervisor and for all the help when everything got confused due to covid, for all the feedback and for inspiring me to choose this subject. All the classes and all the courses that I took helped me and showed me what I would like to do in the future, and everything that I learn through these years were helpful and interesting.

I would like to thanks to all my colleagues from MBHA, they helped through this whole master, for all the laughs and coffee, and specially for letting me in and feel part of something. Thank you for supporting me through this challenge and for hearing me out when I was in doubt, and for being happier than me when I got this huge opportunity. And to all the professors from the master, with a special thank you to Professor Francisco Pina-Martins for helping me with my dissertation and with the R program.

And last thanks to all the springtails that made me company during this amazing adventure and allow me to write this study in order to improve the world. I gave you names and took care of you in the way I could.

Resumo

Neonicotinóides são um dos inseticidas mais usados a nível mundial, sendo que uma grande parte dos mesmos acaba no solo onde acaba por persistir. Os neonicotinóides podem causar espasmos involuntários, paralisia e eventualmente morte. O inseticida usado nesta dissertação, imidacloprida, degrada-se lentamente e tem uma meia vida elevada, acabando por afetar organismos não-alvo no solo, como os Colêmbolos. Inicialmente acreditava-se ser não tóxico para animais invertebrados, no entanto, estudos recentes demonstram que este inseticida consegue causar efeitos adversos nos mesmos. Apesar de ser um neonicotinóide banido do uso regular na União Europeia ainda é usado noutras regiões e uma vez que tem uma elevada meia-vida permanece nos solos durante muito tempo, sendo possível observar os seus efeitos mesmo depois do seu desuso. Afeta múltiplas gerações e os animais quando expostos não desenvolvem resistência ao composto em questão.

É importante compreender como funcionam as comunidades de solo uma vez que estas mantêm a boa qualidade do mesmo. Os colêmbolos são microartrópodes que desempenham um papel fundamental no solo, uma vez que contribuem para a sua decomposição e reciclagem de nutrientes. Deste modo é importante estudar colêmbolos pois estes são bons bioindicadores de poluição.

Os testes de toxicidade baseiam-se na exposição do solo a contaminantes, não permitindo assim a observação detalhada dos indivíduos durante as experiências. Além disso, estes testes não permitem avaliar o que acontece na natureza, quando os organismos do solo podem ser expostos a contaminantes também através da alimentação. Deste modo é necessário estudar esta rota de exposição e criar um método de alimentação eficiente e fiável.

Esta dissertação tem como objetivo desenvolver um método de exposição dos colêmbolos a contaminantes através da comida, permitindo assim a monitorização contínua dos animais durante os ensaios sem ser necessário interrompê-los para recolher e registar os seus efeitos.

Os colêmbolos tendem a migrar em busca de alimentos de melhor qualidade e quando forçados a ingerir comida contaminada acabam por ser seletivos em relação às suas características, dando prioridades a umas em relação a outras.

A espécie normalmente usada para estes testes de toxicidade é *Folsomia candida,* no entanto a mesma não é encontrada facilmente na natureza, estando presente usualmente em cavernas ou vasos. Deste modo, os efeitos dos contaminantes nesta espécie não são, provavelmente, representativos dos milhares de espécies de colêmbolos existentes.

Assim,nesta dissertação foram estudadas duas espécies de colêmbolos, *Folsomia quadrioculata* e *Hypogastrura viatica*.. Ambas têm uma reprodução sexuada, ao contrário de *F. candida* que possui uma reprodução assexuada, têm diferentes traços de vida, habitats e diferentes métodos de adaptação. Sendo que *F. quadrioculata* vive maioritariamente no solo, a maiores profundidades, protegida dos fatores externos, como alterações climáticas ou alterações antropogénicas, e quando obrigada a ingerir comida contaminada, lida com os diversos tipos de stress, enquanto *H. viatica* é uma espécie que se localiza mais à superfície, sendo mais móvel e podendo migrar quando em perigo ou em busca de comida, mas ao mesmo tempo adaptando-se facilmente a mudanças no clima ou até mesmo mudanças antropogénicas.

No ensaio preliminar, para testar as diferentes sensibilidades ao mesmo contaminante, estas duas espécies foram alimentadas com casca de árvore contaminada com imidacloprida e para cada espécie fizeram-se 5 réplicas e uma vez que não é possível distinguir-se o sexo dos animais, cada ensaio continha 20 animais para comparação com os ensaios controlo. De forma avaliar os efeitos do

inseticida nos colêmbolos foram usados animais recém-nascidos em vez de adultos uma vez que estes últimos tendem a ser mais resistentes. Esta experiência teve a duração de 40 dias para *Folsomia quadrioculata* e 50 dias para *Hypogastrura viatica*, uma vez que estas espécies atingem a maturidade aproximadamente ao fim de 25 e 35 dias, respetivamente, dando-nos assim a oportunidade de documentar a deposição de ovos. A comida foi contaminada de duas formas diferentes: mergulhar a casca de *Tilia cordata* numa solução com 129 ug/L de imidacloprida à temperatura ambiente ou humedecer a mesma com uma micropipeta (concentração final estimada de 129 mg/kg de imidacloprida). Tratamentos controlo foram criados, sendo que a casca da árvore também era humedecida ou mergulhada em água destilada e também foi adicionado um tratamento somente com a casca da árvore.

O método mais preciso foi o segundo (micropipeta), uma vez que a concentração final medida foi similar às concentrações nominais, enquanto o método de submersão da comida na solução com imidacloprida indicou valores 38 vezes superiores às concentrações nominais. Os resultados deste estudo demonstram que *H. viatica* é mais sensível a nível de produção de ovos do que *F. quadrioculata*, no entanto tal não se demonstra quando se trata do tamanho corporal das espécies pois este foi afetado negativamente em ambas as espécies.

Uma vez estabelecido o melhor método para monitorizar o efeito da imidacloprida nas duas espécies em estudo, realizou-se um ensaio de concentração-resposta dirigido de modo a estudar os efeitos do neonicotinóide nos diversos traços de vida em *H. viatica* juvenis. Este ensaio foi desenvolvido com 5 diferentes concentrações sub-letais desde 0.01 a 1.2 mg/kg de casca de *T. cordata*. Devido a limitações de tempo em resultado da pandemia COVID-19, que impediu o acesso ao laboratório, apenas foi possível realizar o ensaio com a espécie *Hypogastrura viatica*, uma vez que é possível registar a muda do exoesqueleto e medir o tamanho corporal dos indivíduos, ao contrário da espécie *Folsomia quadrioculata*.

As concentrações escolhidas foram consideradas sub-letais, com exceção da mais elevada que é uma concentração letal, e devidamente escolhidas uma vez que as concentrações usadas durante este estudo estão presentes na natureza.

Os exemplares dascaixas com a concentração mais elevada não se reproduziram e, apenas em duas de cinco caixas na concentrações sub-letais mais elevadas (0.13 e 0.40 mg/kg) é que se reproduziram. Quando expostos a imidacloprida, os colêmbolos tendem a atingir a maturidade sexual mais tarde de forma a darem preferência a outro atributo, como sobrevivência ou até mesmo crescimento.

A exposição de *H. viatica* a concentrações de imidacloprida encontradas no solo fez com que houvesse uma redução na frequência de muda do exoesqueleto e, consequentemente, uma redução no seu tamanho corporal pois os animais despendem mais energia a processar o stress tóxico e em contrapartida menos tempo é dedicado ao seu crescimento. É importante estudar a frequência de muda do exoesqueleto uma vez que esta é uma rota de eliminação de tóxicos, no entanto pouca informação existe sobre a mesma.

O estudo destas características é relevante porque se os colêmbolos atingem a maturidade sexual mais tarde e/ou sofrem um atraso no desenvolvimento corporal, a produção de ovos reduz-se, havendo um impacto negativo nas populações.

Ainda existe pouca informação sobre estas duas espécies de Colêmbolos e especialmente sobre como certos traços de vida são afetados por diversos contaminantes. Deste modo, é necessário no futuro usar este tipo de metodologias menos invasivas que permitam a análise e monitorização das espécies sob ação de inseticidas. É importante também ter em mente que os poucos estudos que existem sobre estas

duas espécies e a sua exposição a imidacloprida são realizados em solo, pelo que quando comparados os resultados, há uma grande diferença, quer de rota, quer de método de aplicação.

Por fim é necessário considerar que, uma vez que a imidacloprida aumenta a idade de maturação dos colêmbolos, futuras experiências devem aumentar o tempo de estudo para que seja possível observar os efeitos a longo termo deste inseticida nos animais.

Palavras-chave: Colêmbolos; Neonicotinóides; Imidacloprida; Método de alimentação; Exposição concentração-resposta.

Abstract

Neonicotinoids are the most widely used insecticides worldwide. A large proportion ends up in the soil, where they degrade slowly, and are likely to affect non-target species in the soil community, such as Collembola (springtails). Springtails play an important role in soil ecosystems by contributing to litter decomposition and nutrient cycling. Standard toxicity tests use soil exposure to toxicants, which does not allow for detailed observation of the individuals during the experiment. These tests are developed to expose organisms through soil to certain types of contaminants, but in nature springtails can also be exposed to them through their diet. We therefore aimed to, and successfully developed, a method for dietary exposure to toxicants, allowing us to observe springtails performance during exposure. The neonicotinoid imidacloprid was applied as a test substance, and we studied two springtail species, *Folsomia quadrioculata* and *Hypogastrura viatica,* with different life histories, habitat, and ecology, to assess potential difference in sensitivity. For each species, five replicates of 20 newly hatched animals were exposed through feed treated with one of two different applications: moistening the feed with a micropipette (estimated final concentration of 129 mg/kg imidacloprid) or soaking it in a solution with 129 ug/L imidacloprid, at room temperature. Moistening the *Tilia cordata* bark was most accurate, giving concentrations similar to the estimated nominal ones, while soaking resulted in 38 times higher concentrations than moistened measured method. With our established method of moistening the bark, we conducted a concentration-response experiment to study how imidacloprid affects different life history traits in juvenile *H. viatica* using 5 sub-lethal concentrations from 0.01 to 1.2 mg/kg dry bark. Due to time constraints as a result of the COVID-19 pandemic, which prevented access to the laboratory, it was difficult to analyze some endpoints for *Folsomia quadrioculata*, and since it was possible to register molting and measure body size in *Hypogastrura viatica* we conducted the second experiment only on that species. Exposure to the soil relevant concentrations of imidacloprid caused *H. viatica* to reach maturity late, reduced its molting frequency, and thus reduced its body size. Since these endpoints have negative impacts on Collembola egg production, the studied neonicotinoid shown negative effects on population growth and may therefore cause population-level effects. There is still little information about these two springtails species and especially on the life history traits for springtails in general. Therefore, in the future it would be important to use methods that allow the monitoring of effects on life history traits.

Keywords: Springtails; Neonicotinoids; Imidacloprid; Dietary method; Concentration-response Exposure.

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Abbreviations

$AgNO_3$	Silver nitrate
ANOVA	Analysis of variance
dH_2O	Distilled water
DRC	Dose response curve
EC_x	Effective concentration affecting a specific % of the population
EU	European Union
LC_x	Lethal concentration affecting a specific % of the population
LL	Log-logistic function
LOD	Limit of detection
LOEC	Lowest Observed Effect Concentration
Log	Logarithm
Max	Maximum
MeCN	Acetonitrile
Min	Minimum
n	Number of samples
NA	Not available
nAChR	Nicotinic Acetyl Choline Receptor
NIVA	Norwegian Institute for Water Research
NOEC	No Observed Effect Concentration
SD	Standard deviation
SOP	Standard operation procedure
Tukey's HSD	Tukey's honest significant different post hoc test
UiO	University of Oslo

1. Introduction

1.1. Soil microarthropods communities

Understanding the soil communities' function and performance is important to maintain good soil quality necessary for food production (Pollierer & Scheu, 2017). Increasingly, the soil tends to be impacted by anthropogenic activity, including the use of insecticides, causing a decline in abundance of terrestrial arthropods (Wise & Lensing, 2019). Soil microarthropods can function as bioindicators of this pollution, since they have a high site-specificity (Fountain & Hopkin, 2004), and are sensitive to the effects of soil pollutants (Lin *et al.*, 2019; Vanhée & Devigne, 2018), as they are adapted to specific soil conditions (Menta *et al.*, 2019). These organisms are important since they are involved in nutrient cycling processes and maintain soil quality and health (Stork & Eggleton, 1992).

1.2. Springtails of ecological relevance

Springtails (Collembola) are microarthropods (0.5 – 2 mm) that are important parts of the soil community (Moore *et al.*, 2009; Rusek, 1998). Springtails affect soil functioning through the nutrient turnover rates and organic matter decomposition (Krab *et al.*, 2019). They have a high abundance and diversity (Gillet & Ponge, 2005; Vanhée & Devigne, 2018), move in groups and their impact on the soil may vary according to their depth (Eisenhauer *et al*., 2011). Springtails are considered generalist feeders on fungi, algae, detritus, and cyanobacteria (Čuchta *et al.*, 2019; Moore *et al.*, 2009; Scheu & Folger, 2004), and their food preferences will depend on the species and food availability (Hopkin, 1997; Rusek, 1998). Some springtails function as secondary decomposers (Scheu & Folger, 2004; Schnug, Leinaas, *et al.*, *2014*).

As springtails influence microbial ecology and soil fecundity (Čuchta *et al.*, 2019), it is important to understand the impacts of contaminants on the springtails by studying their life-history traits (Knoepp *et al.*, 2012), including mortality and recruitment of juveniles. Traditional toxicity tests are developed for soil exposure to pesticides (ISO, 2011; ISO, 2014; OECD, 2009), but in nature, springtails can also be exposed to environmental contaminants through their diet. Therefore, obtaining more knowledge on the latter exposure route is important to understand the contamination dynamics in soil.

The springtail species most commonly studied is *Folsomia candida* Willem 1902 (Fountain & Hopkin, 2004), and the standard tests cover the endpoints mortality, recruitment, and avoidance behavior (de Lima e Silva *et al.*, 2021; Filser *et al.*, 2014; Lee *et al.*, 2019). *F. candida* is absent from many natural habitats, being found in caves and housepots, and reproduce asexually (de Lima e Silva *et al.*, 2021; Moore *et al.*, 2009). Therefore, the effect on *F. candida* is likely not representative of all the several thousand species within the springtail taxon (de Lima e Silva *et al.*, 2021). In this dissertation, we studied two different species of springtails, *Folsomia quadrioculata* and *Hypogastrura viatica* (Tullberg, 1872), both of which reproduce sexually and cover a wide geographical distribution in the northern hemisphere (Convey *et al.*, 1999; Hughes *et al.*, 2017; Jensen *et al.*, 2006). The two species represent different habitats in depth: *F. quadrioculata* is a soil litter-dwelling springtail that is very sensitive to dry and low humidity environments (Hertzberg & Leinaas, 1998), while *H. viatica* is a surface-dwelling springtail resistant to extreme conditions and very mobile (Hertzberg *et al.*, 2000). Developing a more efficient method for two different species ensures robustness and can thus likely be applied to several other springtail species.

There is little information about both species' response to toxic stress and how contaminants affect different individuals from different habitats. Soil-dwelling springtails spend their life in the soil, they are more protected from rapidly changing weather conditions, but less mobile and thus less able to

avoid contamination. Soil litter-dwelling and surface-dwelling species spend their life partially or entirely on the surface being more in contact with changes in their habitats (de Lima e Silva *et al.*, 2021). Different species can have different effects when exposed to the same contaminant (Frampton *et al.*, 2006).

1.3.Life history traits

Insecticides can affect life history traits of organisms causing adverse effects depending on the concentration, exposure time or method of exposure of the insecticide in question (Sengupta *et al.*, 2020). Thus, it is important to study the different life history traits (Siepel, 1994) given the generation of defense patterns by springtails when subjected to a polluted environment (Malmström, 2012). Springtails end up with lower fitness in polluted soils with inferior-quality food and therefore must adapt behaviorally and physiologically to maintain equilibrium (Jensen *et al.*, 2006). These animals tend to migrate in search of better-quality food or trade-off to prioritize one attribute over the other, i.e., preferring to survive rather than reproduce (Fountain & Hopkin, 2004). The optimal strategy depends on the food available, environmental factors, and life history traits of the specific species (Jensen *et al.*, 2006).

Ecotoxicological experiments using springtails are carried out in soils (Greenslade & Vaughan, 2003; OECD 2009; Zhang & Qiao, 2020) or in microcosms (Sengupta *et al.*, 2017; Sjursen *et al.*, 2005), in which the animals are hidden within the soil. Thus, there is a need to develop a method where the animals can be monitored during exposure.

One of the commonly studied endpoints and life history traits of springtails is recruitment, which is considered a sensitive trait for toxic effects. Recruitment is the number of offspring produced and survived throughout the experiment (Schnug, Leinaas, *et al.*, 2014) while reproduction is the number of offsprings that were produced (OECD 2009). However, the sensitivity of individuals to toxic substances depends on their developmental stages (Lee *et al.*, 2019). Springtails' molting and thus growth, are negatively affected by contaminants (de Lima e Silva *et al.*, 2021). Their fertility depends on the survival and body size, and if they reach maturity at a later age, or with a smaller body size than optimal, the production of eggs will decrease (Ernsting *et al.*, 1993). Consequently, reduced egg production will cause reduced population growth.

Springtails tend to avoid the food or stop eating, when they are in the presence of contaminated food (Scheu & Folger, 2004). With food restriction, springtails tend to molt their exuviae less often and in turn grow slower, thus preventing them from reaching the ideal body size to reproduce when expected (Joosse & Veltkamp, 1969). Springtails molt throughout their entire life (Birkemoe & Leinaas, 2000). In addition to growth, molting has thus an excretion function (Humbert, 1979). During molting, the intestinal epithelium is also excreted and renewed, in which the excreted contains contaminants ingested through food (Joosse & Buker, 1979; Schnug, Leinaas, *et al.*, 2014; Zhang & Qiao, 2020). Some organic pollutants tend to negatively affect the molting process of springtails and consequently their growth (Lee *et al.*, 2019; Zhang & Qiao, 2020), although there are not many studies on this endpoint. It is important to study the molt process as reduced excretion can cause reduced health, but also because hatchlings are more sensitive to contaminants, making them more susceptible to be affected in terms of growth and thus reproduction. To properly study these responses and life history traits, the springtails need frequent observations, which is not possible when maintained in soil.

1.4. The neonicotinoid imidacloprid

One of the most used insecticide groups worldwide are neonicotinoids, and a large proportion of the active ingredient ends up in the soil where their persistence is high (Goulson, 2013). Residues of neonicotinoids can elicit adverse effects on non-target soil organisms, such as springtails. Neonicotinoids act as a nicotinic acetylcholine receptor (nAChR) agonist as they compete with acetylcholine neurotransmitters to bind and activate the nAChR. The binding causes overexcitation of the neurons (Burke *et al.,* 2018; Menon & Mohanraj, 2018), causing involuntary spasms, paralysis, and eventually death (van Gestel *et al.,* 2017). The neonicotinoid imidacloprid is one of the most used insecticides all over the world (Bandeira *et al.,* 2020). It has been used in agriculture as a protection against certain pests (de Lima e Silva *et al.,* 2017), for household use and it was utilized to protect several trees (Crayton *et al.,* 2020). Imidacloprid was first assumed non-toxic to certain types of animals (Suchail *et al.,* 2000), but has been shown to be toxic to honeybees (*Apis mellifera* L, 1758*)* (Schmuck *et al.,* 2001), white-tailed deer (*Odocoileus virginianus* Zimmermann, 1780) (Berheim *et al.,* 2019) and salamanders (*Desmognathus spp)* (Crayton *et al.,* 2020). Imidacloprid's half-life has a wide range, from 28 to 1250 day, depending on soil type and other environmental factors (van Gestel *et al.,* 2017). Imidacloprid is used only on greenhouses all over Europe since 2018 (Regulation (EU) No 2018/783; Cressey, 2017). Due to its long half-life, it can be found in the soil for several years (Sengupta *et al.,* 2020) causing effects on the organisms. The effects of this contaminant can be seen for several generations and the animals do not develop resistance against the component (van Gestel *et al.,* 2017).

1.5. Aims

The overall aim of this project was to study the effects of imidacloprid on life-history traits in springtails exposed through diet. As dietary exposure to contaminants in soil organisms is rarely studied, and the method was thus in need of development. Two different spiking methods were tested in order to do so. The objectives (denoted 1 and 2) and associated hypothesis (denoted with letters) of the method development, and the subsequent study of sub-lethal effects by imidacloprid were:

1. Develop a suitable method for dietary exposure subjecting two ecologically relevant springtail species to imidacloprid.
    a. Different spiking methods will result in a similar concentration in the feed and cause similar effects within each species.
    b. The level of effects from imidacloprid exposure will be different in *Hypogastrura viatica* compared to *Folsomia quadrioculata*.

2. Study effects of dietary exposure to range of sub-lethal imidacloprid concentrations on life-history traits in *Hypogastrura viatica*.
    a. Age at first reproduction will increase with higher concentrations of imidacloprid.
    b. Body size at first reproduction will decrease with higher concentrations of imidacloprid.
    c. With increasing imidacloprid concentration, molting frequency will decrease, and subsequently cause reduced body size.
    d. For control animals there will be a positive relationship between age and body size at first reproduction, but there will be no relationship for the other concentrations of imidacloprid exposure.
    e. Molted exuviae and body size will have a directly proportional positive relationship within each treatment.

2. Materials and methods

2.1. Study species

Cultures of springtail species *Folsomia quadrioculata* and *Hypogastrura viatica* were provided by Professor emeritus Hans Petter Leinaas from the Department of Biosciences, at the University of Oslo, Norway. They were cultured for several years so do not have any maternal/paternal or environmental effects. Both species were collected from Svalbard, Norwegian Arctic, *F. quadrioculata* was collected in Little State Island (81°N, 20°E) and *H. viatica* in the bay Fjortende Julibukta (79°N, 12°E).

F. quadrioculata (Figure 2.1) is soil litter-dwelling (hemi-edaphic) springtail and an abundant species in many types of habitats (Sengupta *et al.*, 2016), widely distributed in temperate and artic regions (Sengupta *et al.*, 2017). It is dependent on habitat coverage and is very sensitive to dry and low humidity environments (Hertzberg *et al.*, 2000).

Figure 2.1- Folsomia quadrioculta in test containers filled with a mixture of plaster of Paris and charcoal. Picture taken by Silje Marie Kristiansen.

H. viatica (Figure 2.2) is a surface-dwelling (edaphic) springtail, widely distributed in the northern hemisphere, and in the south hemisphere through human activity (Bello *et al.*, 2019; Hertzberg *et al.*, 2000; Jensen *et al.*, 2006), and can be found usually in coastal habitats or close to the shore or in habitats influenced by saline (Hughes *et al.*, 2017). It is resistant to extreme conditions of drought and very mobile (Hertzberg *et al.*, 2000).

Figure 2.2 - Hypogastrura viatica in dry bark from a culture box. Picture taken by Silje Marie Kristiansen.

The springtail cultures were kept in cylindrical containers with a radius of 1.5 cm and a height of 3.5 cm, with a 0.5-1.0 cm high layer of mixture of plaster, darkened with a little activated carbon. The plaster can obtain water and therefore maintains the optimal moisture conditions that are required for the survival of the springtails. The springtails were fed with small pieces of dry bark covered with a layer of cyanobacteria, gathered on *Tilia cordata* trees from the University campus near the lab. The bark was defaunated overnight at -80°C. The bark for the cultures was replaced once a week, and a few drops of distilled water was added to maintain the moisture on the boxes. All cultures were kept in incubators at a temperature of 15°C since it is a temperature known to work best for the cultures.

2.2.Method development for dietary exposure

The insecticide used in both experiments was the neonicotinoid imidacloprid (N- [1 - [(6-Chloro-3-pyridyl) methyl] -4,5-dihydroimidazol-2-yl] nitramide) produced by Sigma-Aldrich with a purity of ≥ 98.0 % and CAS number 138261-41-3. The imidacloprid was dissolved in distilled water to obtain a concentration of 129 µg/L, being the only concentration used in the method development. The concentration of 129 µg/L of imidacloprid (equivalent to the solution used to obtain a field-realistic dose of 0.03 mg/kg dry soil) was chosen, as previous studies with soaked bark showed that this concentration is sub-lethal, which negatively affects reproduction rate (Kristiansen et al., 2021; Sengupta *et al.*, 2020).

The two springtail species were exposed to imidacloprid through feed treated with one of two different applications: (I) moistening the food with a micropipette (Tourinho *et al.*, 2015) or (II) soaking the food overnight in a solution with imidacloprid. The bark with cyanobacteria was removed from the *Tilia cordata* branches in similar thin layers, and randomly divided into 5 containers, one for each treatment (or control). The bark in two of the containers were cut into pieces of 25 mg. The treatments and controls were prepared as follows:

i) dry bark (control); pieces of bark that was not processed after being defaunated.

ii) soaked bark (control); bark was soaked overnight in distilled water,

iii) moistened bark (control); each piece of 25 mg bark was moistened with 25 µL distilled water,

iv) spiked soaked bark; bark was soaked overnight in a solution of 129 µg/L of imidacloprid,

v) spiked moistened bark; each piece of 25 mg bark was moistened with 25 µL imidacloprid solution obtaining a final concentration of 129 µg/kg dry bark.

After preparation, the bark was left to dry at room temperature, and kept dark to avoid degradation of imidacloprid. To determine whether spiking method (soaked or moistened bark) affected the true concentration in the bark, approximately 1 g bark from treatments ii-v were homogenized separately and analyzed for imidacloprid by the Institute for Water Research (NIVA) in Oslo as presented in section 2.4.

Each treatment had 5 replicates per species, consisting of approximately 20 hatchlings, less than 24 hours old. The eggs were laid at a temperature of 15°C and when hatched they were moved into the boxes at a temperature of 20°C. There is no knowledge that this change between temperatures in hatchlings have any effect of acclimatization on the animals and since this exchange was made in all the boxes for all the eggs there is no variation on the experiment. The experiment had a duration of 40 days for *F. quadrioculata* and 50 days for *H. viatica*. These durations were chosen because at 20°C, *F. quadrioculata* and *H. viatica* mature (first reproduction) after approximately 25 and 35 days, respectively, and this would allow us to document egg production for approximately 2 weeks. The feed was replaced every 3 days, to avoid fungi growth which is more frequent at this temperature.

The experimental design is illustrated in Figure 2.3.

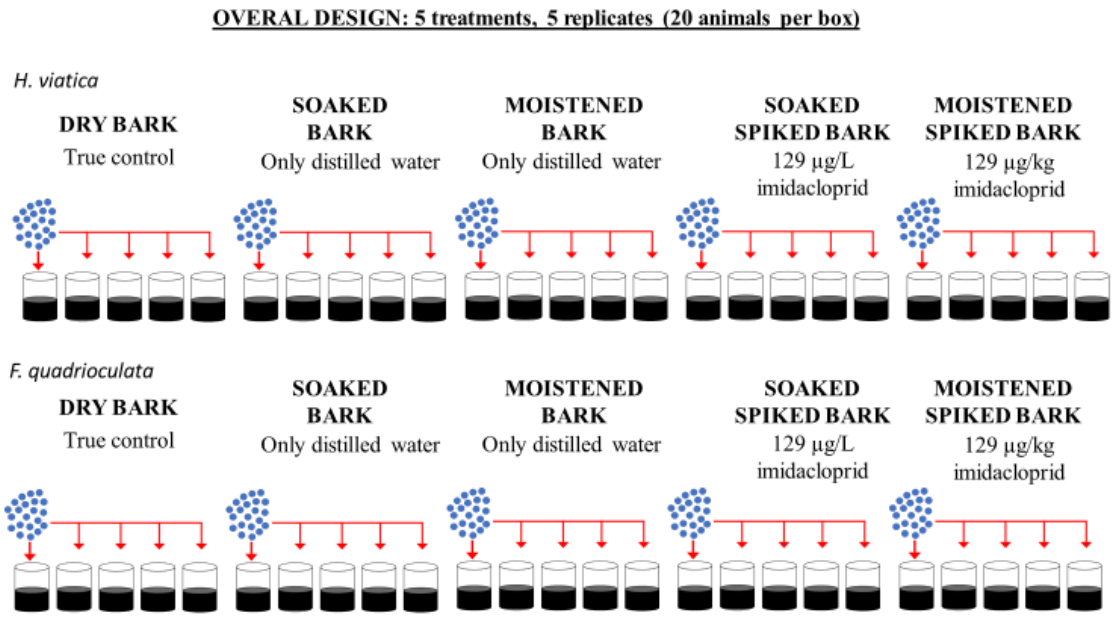

Figure 2.3 - Experimental design for the method development of dietary exposure to imidacloprid for springtails.

The endpoints measured were survival, age at first reproduction, body size at the end of the experiment, and egg production. Reproduction is a suitable endpoint to determine the health of the animals during the experiment because it is known that healthy springtails reproduce frequently (personal experience).

Sexes of living *F. quadrioculata* and *H. viatica* cannot be distinguished, so every box contained 20 animals to ensure enough individuals of each sex, and therefore ensure reproduction, instead of 10 which is recommended in the reproduction standard operating procedure (SOP) for *Folsomia candida* (ISO 11267:2014; OECD 2009). The juvenile springtails are difficult to spot because of their light color and high activity level, and it is therefore common to lose some individuals during an experiment (Table 2.1).

Table 2.1 - Experimental development with Hypogastrura viatica and Folsomia quadrioculata where it was registered the number of animals in the beginning of the experiment, animals harvested in the end of the experiment, animals that were dead or lost during the experiment and the number of animals used to measure the body length and egg production (animals for endpoints registration).

Species	Treatment	BOX ID (replicate)	Animals in the beginning	Animals harvested	Lost and Dead animals	Animals for endpoints registration
Hypogastrura viatica	**Dry bark**	1	20	17	3	10
		2	20	16	4	14
		3	20	19	1	11
		4	20	16	4	16
		5	20	20	0	16
	Soaked bark, control	6	21	21	0	8
		7	20	16	4	11
		8	20	18	2	11
		9	20	15	5	9
		10	20	17	3	16
	Moistened bark, control	11	20	18	2	15
		12	20	18	2	13
		13	21	21	0	12
		14	20	16	4	12
		15	20	16	4	14
	Soaked bark, spiked	16	20	0	20	0
		17	20	0	20	0
		18	20	0	20	0
		19	20	0	20	0
		20	20	0	20	0
	Moistened bark, spiked	21	20	12	8	10
		22	20	5	15	5
		23	20	16	4	13
		24	20	16	4	13
		25	20	20	0	21
Folsomia quadrioculata	**Dry bark**	26	20	19	1	15
		27	20	18	2	15
		28	20	14	6	14
		29	20	13	7	13
		30	20	16	4	16
	Soaked bark, control	31	20	20	2	20
		32	20	15	5	15
		33	20	15	5	15
		34	20	17	3	16
		35	21	21	0	20
	Moistened bark, control	36	20	19	1	16
		37	20	14	6	9
		38	20	9	11	9
		39	20	16	4	14

		40	20	12	8	12
	Soaked bark, spiked	41	20	0	20	0
		42	20	0	20	0
		43	20	0	20	0
		44	20	0	20	0
		45	20	0	20	0
	Moistened bark, spiked	46	20	14	6	10
		47	20	17	3	16
		48	20	4	16	4
		49	20	15	5	13
		50	20	14	6	12

During the experiment, the dead animals were removed and counted. At 40 and 50 days for *F. quadrioculata* and *H. viatica*, respectively, the animals in each box were harvested in 70% ethanol, which was heated to 70ºC to allow the animals to stretch. The animals were counted and stored until body length analysis. The body length measurements were carried out using the image software Leica Application Suite. This software consists of capturing images of the animals when viewed under a microscope and then the program allows us to measure the length of the animals' bodies (Figure 2.4).

Figure 2.4 - Measurement of body size on Folsomia quadrioculata, using Leica Application Suite software. Author´s original.

2.3. Concentration-response exposure

The experiment for sub-lethal effects of imidacloprid on springtails was performed on *H. viatica*, using the most optimal dietary exposure method determined in the previous experiment, moistening the bark with a micropipette.

In this experiment we exposed *H. viatica* to imidacloprid through bark. The following imidacloprid concentrations were applied: 0.01, 0.04, 0.13, 0.4, and 1.2 mg/kg dry bark, in addition to control bark (0 mg/kg dry bark), which was moistened with distilled water. Concentrations of imidacloprid reported in nature usually within the range of 0.012 to 0.018 mg/kg soil, and the highest reported concentration in soil is 0.6 mg/kg dry soil (Anderson & Harmon-Threatt, 2019). This means that the concentrations applied were field-realistic, with the exception of the highest nominal concentration.

Defaunated bark was removed from the branches in thin layers, divided randomly into 6 containers, one for each treatment. The bark was cut into pieces of 25 mg and moistened with 25 µL solution of the respective concentration (approach v from the first experiment). Each concentration had 5 replicates, except control that had 9 replicates consisting of approximately 20 hatchlings, less than 24 hours old. Due to an error, 4 control boxes were placed at 15°C for one day, so to eliminate bias temperature effect, 4 extra experimental control boxes were included. The experiment was run at 20°C, and lasted until the animals reached maturity, i.e., each replicate was terminated when the first eggs were observed. The bark was replaced every 3 days. As imidacloprid is expected to negatively affect reproduction, thus potentially delaying maturity, or hindering reproduction completely, replicates that did not reproduce were harvested when reaching 1.5 times the age at maturity for controls.

The experimental design is illustrated in Figure 2.5.

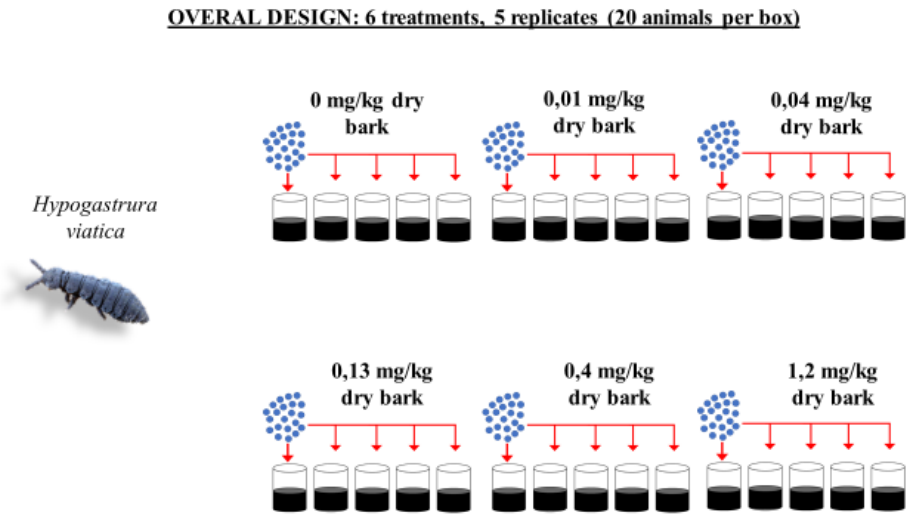

Figure 2.5 - Overall design of the second experiment, concentration-response toxicity of imidacloprid to *Hypogastrura viatica* using moistened spiked bark. Each treatment had 5 boxes, except control (0 mg/kg dry bark) with 9 boxes, and each box had approximately 20 animals. The control was added only distilled water (dH_2O).

The measured endpoints included survival, age at first reproduction, body size and molted exuviae, as described for the method development. At every check-up, any dead animals were registered before removed. The number of molted exuviae and the number of animals were counted, providing molts per animal at each time point. The animals that survived until time of termination were harvested and analyzed for body length in the same manner as explained for the first experiment (section 2.2).

2.4. Chemical analysis of food

To verify that the imidacloprid concentrations used in this experiment were similar to the nominal concentrations, chemical analyses of the bark was conducted by NIVA. Randomly selected bark from each treatment were homogenized using a hand mixer and divided on three replicates. NIVA extracted imidacloprid with acetonitrile (MeCN) and conducted the chemical analysis using high-performance liquid chromatographic-mass spectroscopic analysis. The detection limit (LOD) is 0.1 ng/g imidacloprid or 0.0001 mg/kg imidacloprid. The full method is described in Sengupta et al., 2020.

2.5. Data treatment and statistical analyses

All statistical analyses and graphics were performed with *RStudio Team (2019)*, with the significance level set to 5%. Data was determined to meet the test assumptions of normality and homoscedasticity using Shapiro Wilk's test and the Levene's test. If the criteria were not met, data was transformed using a square root or log transformation to fulfill the assumptions. If the assumptions were still not met, non-parametric tests were used, e.g., Kruskal Wallis test.

For the method development experiment, the survival data was analyzed using the R-package *survival* (Therneau & Grambsch, 2000) that uses Kaplan-Mayer statistics which analyze "time-to-event" data. Thus, it was possible to use the dead and lost animals throughout the experiment since this allows to "censor" the lost data. A log-rank test was performed to determine if there were differences between the survival curves in the different treatments.

Egg production was presented as cumulative number of eggs per animal. As the date for lost animals was unknown, the lost animals were treated as lost in the middle of the experiment (20 days for *F. quadrioculata* and 25 days for *H. viatica*). Differences in age at first reproduction for *H. viatica,* egg production and body size between treatments were analyzed using analysis of variance (ANOVA), followed by a Tukey's honestly significant difference test if significant. Due to the low amount of data for *H. viatica*, only 2 boxes reproduced, a statistical analysis for the age at first reproduction was not performed.

For both experiments, the age at first reproduction was defined as the average between the two observations, i.e., age in number of days, where eggs were first found. Body size, in mm, was measured for each animal since each box had a different number of organisms that survived. When combined, body size for the different treatments do not show the real differences. In the concentration-response exposure experiment the body sizes for the first box were not measured since the harvested animals were not suitable for reliable measurements, having dried in.

For concentration-response exposure, all the endpoints were observed and analyzed using a dose-response curve, *Drc* package in R (Ritz *et al.,* 2015). The data for mortality, age, and body size at first reproduction, molted exuviae and the different relationships between endpoints did not meet the requirements of any explored models (Appendix, section 8.2). Dietary descriptors estimating the lethal concentration and effective concentration affecting a specific percentage of the population (LCx and ECx, respectively) could not be calculated, as it was not possible to fit a model to the data. Therefore, the No Observed Effect Concentration (NOEC) and the Lowest Observed Effect Concentration (LOEC) were determined instead. Since survival could not be transformed (Shapiro test: p-value= 9.458e-07, Levene test: p-value= 0.0028) and did not meet the assumptions for an analysis of variance (ANOVA), a Kruskal-Wallis rank sum test was performed, followed by a Pairwise Wilcox test. For the two boxes that did not reproduce an estimated time of 70 days were set as age at first reproduction. This was chosen as an estimate by adding 10 days to the greater age at first reproduction documented, which was 60 days. Differences in age at first reproduction and molted exuviae between concentrations were studied using ANOVA, followed by a Tukey's HSD if significant. For body size at first reproduction, since the data does not follow the assumptions for an ANOVA and could not be transformed (Shapiro test: p-value= 0.0594, Levene test: p-value= 0.0028), a Kruskal-Wallis rank sum test was performed, followed by a Pairwise Wilcox test.

When comparing the age at first reproduction with the body sizes at first reproduction, the median body size of each experimental box was used. The relationship between age at first reproduction and median body size at first reproduction was analyzed using a linear model. Using the mean body size at

first reproduction gave a similar relationship trend within each treatment as using the median (Appendix, section 8.2: Figure 8.5).

The number of molts per animal were divided by the total number of days the animals were monitored. When comparing the molted exuviae with the body sizes, not only were the body sizes at first reproduction used, but also the body sizes of the animals which not reproduced. These animals were also measured for body size at the day of harvest and can thus be applied to study molting. The relationship between body size and molted exuviae was explored using a linear model.

Box no. 5 from control was excluded since it was considered an outlier, reproducing first after 63 days, and is considered abnormal (Kristiansen et al, unpublished), as mean age at first reproduction is 36.5 days (SD= 3.5, min=33, max=45, n=10), for this Arctic population of *H. viatica* at 20°C.

3. Results

3.1. Method Development for Dietary Exposure

3.1.1. Chemical analysis of bark

The measured concentration in the moistened bark was slightly increased, but close to the nominal concentration (sample B4 in Table 3.1). The soaked bark had a measured concentration of imidacloprid that was more than 38 times higher than for moistened bark (sample B3 in Table 3.1). Therefore, bark left overnight in an imidacloprid solution will soak up a high concentration of imidacloprid from the water, making an estimation of nominal concentration difficult. A low concentration of imidacloprid was found in the control samples but could be explained by instrumental residue during analysis. During the experiment, fungi growth was observed to be more frequent in the soaked bark, and the animals appeared to not grow and developed in the same manner as the other treatments. These observations were in agreement with the high difference in concentrations measured between moistened and soaked bark.

Table 3.1 - Concentrations of imidacloprid measured by NIVA in bark treated with distilled water (control) or imidacloprid.

Sample ID	Treatment	Nominal concentration (mg/kg)	Measured concentration (mg/kg)[2]
B1	Control	0	0.0014
B2	Control	0	0.0003
B3	Soaked spiked Method	NA[1]	8.03
B4	Moistened spiked Method	0.129	0.207

[1] NA = not available. This concentration cannot be predicted.
[2]Limit of detection (LOD) = 0.0001 mg/kg.

3.1.2. Survival

All three controls had high survival (more than 90%) for both species, and thus met the quality assurance criteria given in standardized toxicity tests (OECD, 2009), where it states that mortality at the end of the experiment should not be more than 20% in the controls. Exposure to imidacloprid with

moistened bark was sublethal, with a high survival with more than 95% for *F. quadrioculata* (Figure 3.1.a) and 85% for *H. viatica* (Figure 3.1.b). Imidacloprid in the soaked bark had a lower survival in both species (Figure 3.1), with a survival percentage more than 45% for *Folsomia quadriculata* and a more than 25% for *Hypogastrura viatica.* The different treatments are significantly different in terms of survival for both species (log-rank test: p-value<0.0001).

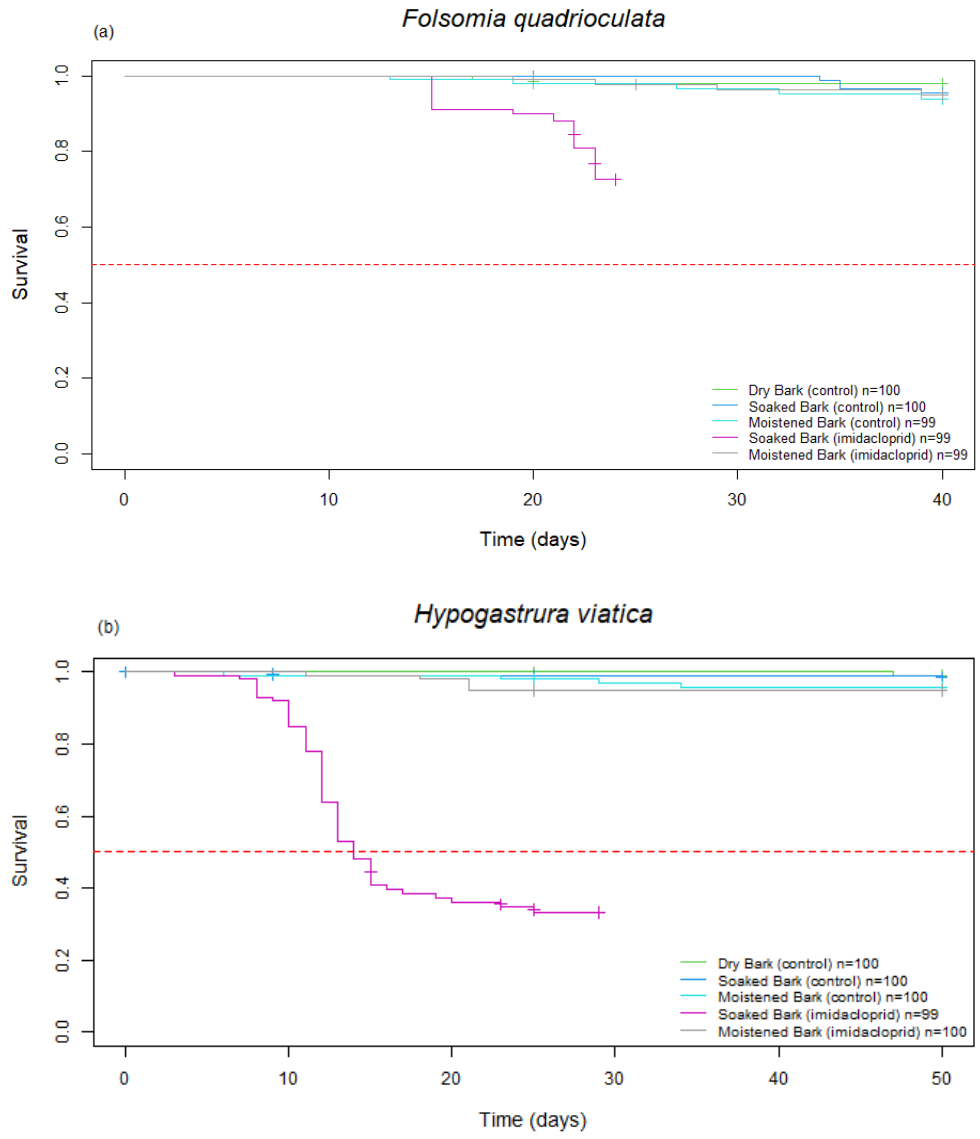

Figure 3.1 - Proportions of survival for two species of springtails, a) Folsomia quadriocuata and b) Hypogastrura viatica exposed dietary to imidacloprid through two different spiking methods, soaked bark, and moistened bark. The red line marks the survival proportion of 0.5.

3.1.3. Age at first reproduction

There was no difference in age at first reproduction between treatments for *F. quadrioculata* (ANOVA, p-value= 0.201, f= 1.728, Figure 3.2.a). While all *F. quadrioculata* replicates exposed to imidacloprid on moistened bark laid eggs, only two *H. viatica* boxes laid eggs within the duration of

the experiment. Therefore, it was not possible to conduct statistical analysis given the scarcity of data for age at first reproduction in *H. viatica* (Figure 3.2.b).

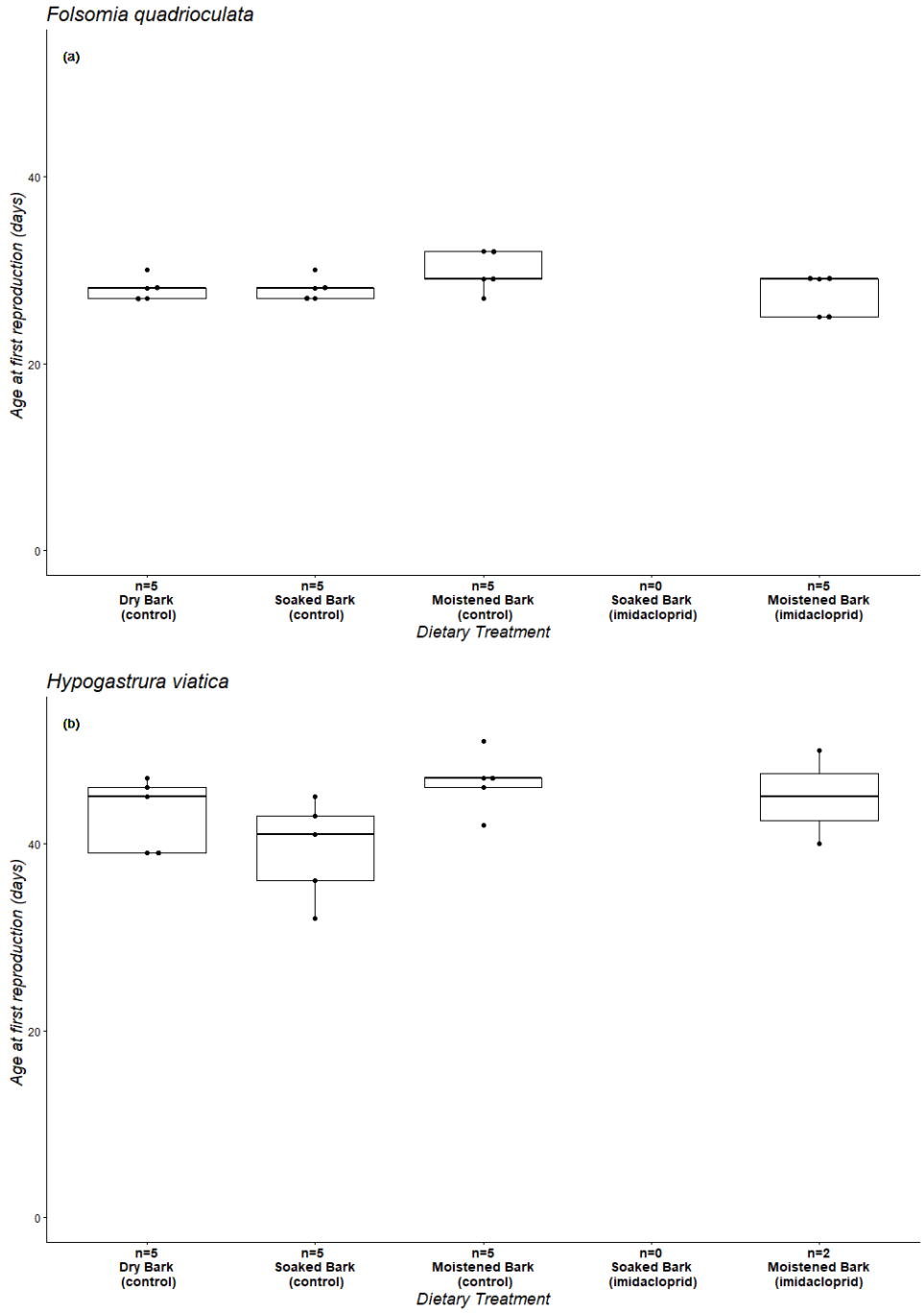

Figure 3.2 - Age at first reproduction for a) Folsomia quadrioculata and b) Hypogastrura viatica, exposed dietary to imidacloprid through two different spiking methods. Data presented as median, quartiles and 10-90 percentiles. • age at first reproduction for each replicate.

3.1.4. Egg production

Egg production was unaffected by imidacloprid for *F. quadrioculata* (Figure 3.3.a, ANOVA, p-value=0.513, f= 0.798), while the cumulative egg production was reduced in *H. viatica* exposed to

moistened spiked bark when compared to dry bark control (Tukey's HSD: p-value < 0.01) and soaked bark control (Tukey's HSD: p-value < 0.001, Figure 3.3.b). No difference was found between the three controls (Tukey's HSD: p-value>0.05).

Tukey HSD results are presented in Appendix, section 8.1: Table 8.1.

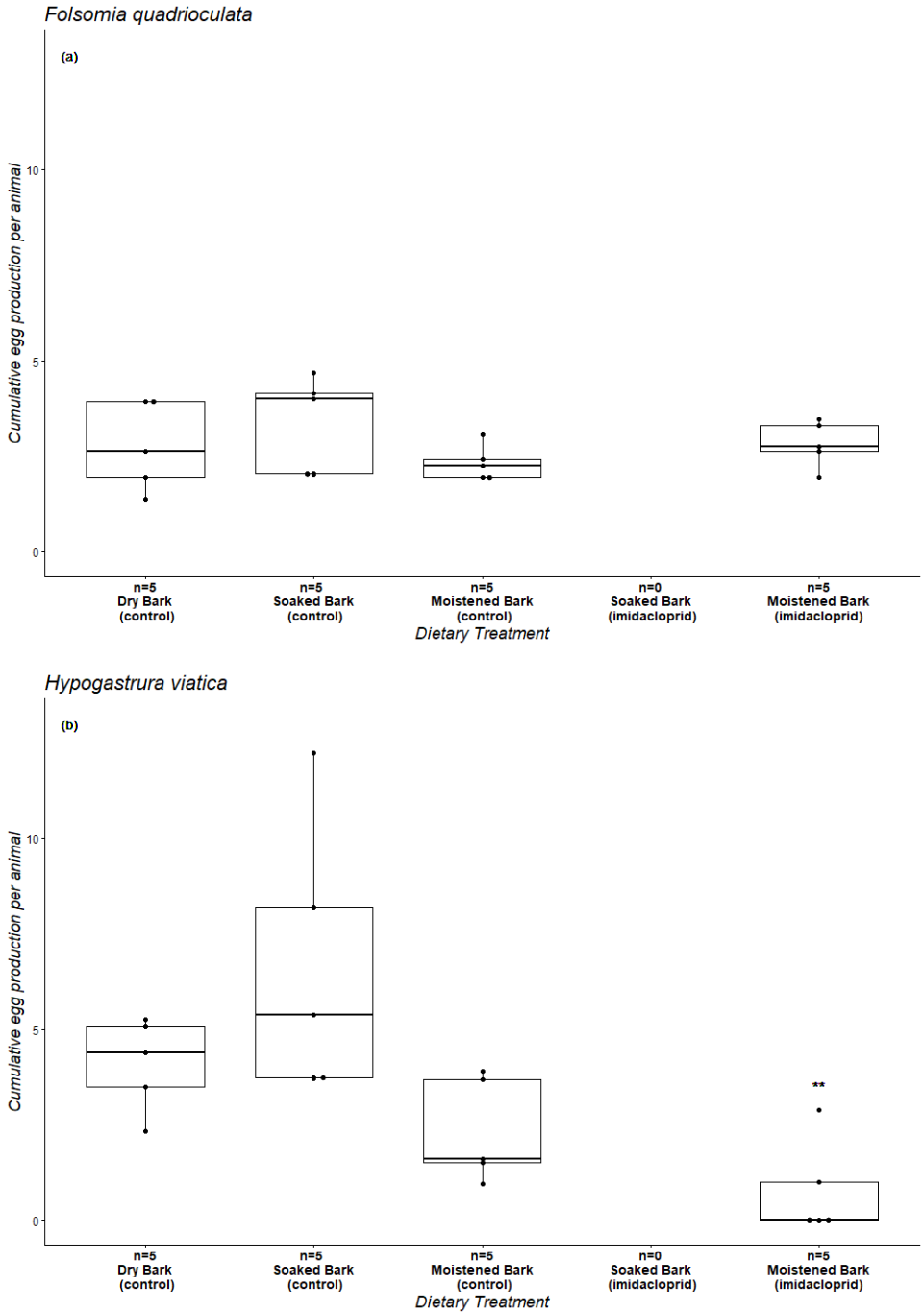

Figure 3.3 - Cumulative egg production for a) Folsomia quadrioculata and b) Hypogastrura viatica, exposed to imidacloprid. Data presented as median, quartiles and 10-90 percentiles. (**) represents significant level p > 0.001 in comparison with the controls (Tukey's HSD).

3.1.5. Body size at 40 days for *Folsomia quadrioculata* and 50 days for *Hypogastrura viatica*

Imidacloprid in moistened bark resulted in lower body size at age 40 days for *Folsomia quadrioculata* (ANOVA, p-value<0.0001, f= 15.65, Figure 3.4.a) and age 50 days for *Hypogastrura viatica* (ANOVA, p-value<0.0001, f= 7.479; Figure 3.4.b), compared to all controls. No difference in body size was found between the three controls (p-value>0.05).

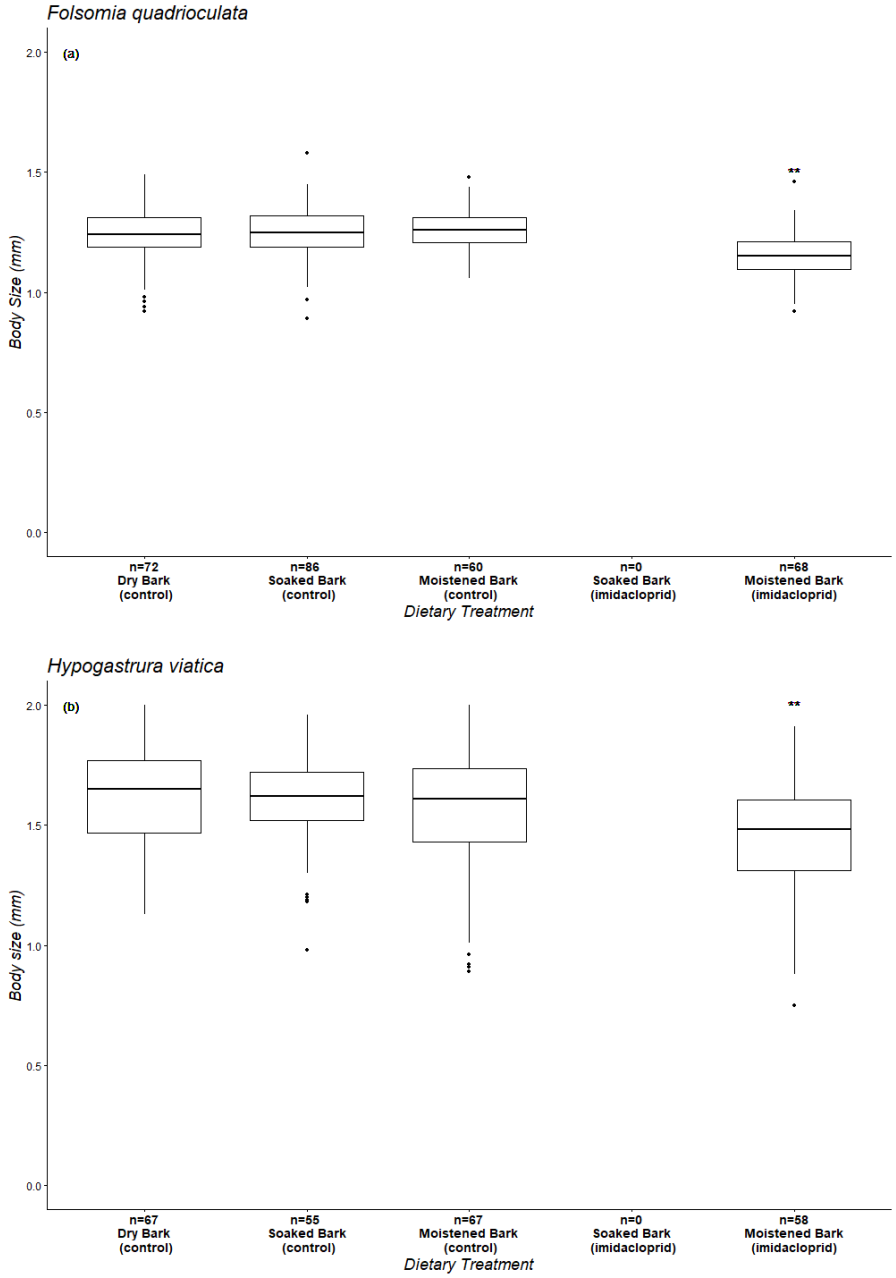

Figure 3.4 - Body size (mm) of a) Folsomia quadrioculata at 40 days, and b) Hypogastrura viatica at 50 days that were dietary exposed to imidacloprid. Data presented as median, quartiles, 10-90 percentiles and outliers. (**) represents significant level p > 0.001 in comparison with the controls (Tukey HSD test).

Tukey post hoc results are presented in Appendix, section 8.1: Table 8.2 and Table 8.3, for more information.

3.2. Concentration-response experiment

3.2.1. Chemical analysis

The chemical analyses of imidacloprid confirmed that bark was spiked in a reliable manner, with measured concentrations near the nominal, and with low variation among replicates (Table 3.2).

Table 3.2 – Nominal and measured concentrations of imidacloprid in bark applied in dietary exposure experiment. The chemical analysis was conducted by NIVA. n=3 per treatment; Detection limit (LOD) is 0.0001 mg/kg.

Nominal concentration (mg/kg dry bark)	Mean measured concentration (mg/kg dry bark)	Standard Deviation (mg/kg dry bark)
0	0.0005	0.0004
0.01	0.0135	0.0009
0.04	0.041	0.0038
0.13	0.112	0.019
0.4	0.355	0.028
1.2	0.95	0.060

3.2.2. Mortality

The imidacloprid concentrations were in the sub-lethal range for *H. viatica* (0-27% minimum and maximum mortality), except for the highest concentration of 1.2 mg/kg dry bark, which was lethal (mortality rate higher than 55%; Figure 3.5). The control (0 mg/kg) has less than 20% mortality when looking at the higher value. The higher concentration had a significant difference from the other treatments (Pairwise Wilcox test: p-value<0.05).

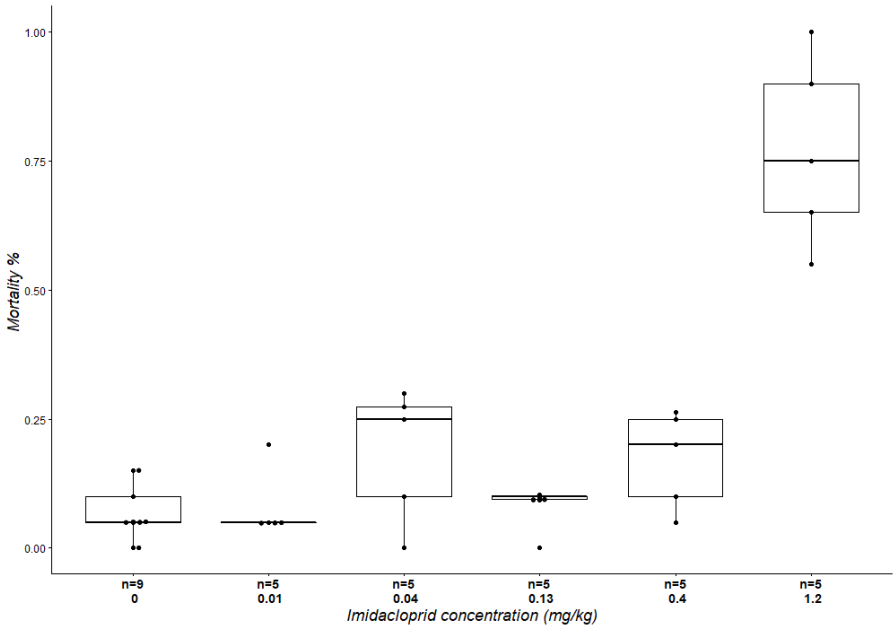

Figure 3.5 - Cumulative mortality percentage of Hypogastrura viatica when exposed to imidacloprid concentrations. Data presented as median, quartiles, 10-90 percentiles and outliers.

A Kaplan-Mayer method was also used to analyze the survival method (Appendix, section 8.2: Figure 8.1).

3.2.3. Age at first reproduction

The age at first reproduction for *H. viatica* was unaffected at all concentrations, except for 0.4 mg/kg dry bark (Tukey HSD: p-value=0.035), with a mean difference of 13 days when compared with the control (0 mg/kg dry bark, Figure 3.6). At the time of termination, reproduction was only found in 4 out of 5 boxes at the higher concentrations of 0.13 and 0.4 mg/kg. The two outliers with late age at these concentrations are assumed age at first reproduction.

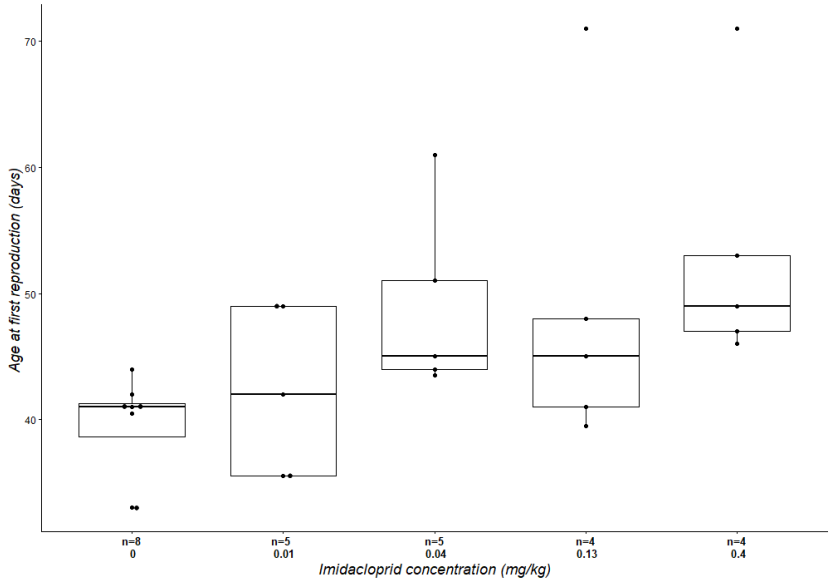

Figure 3.6 - Age at first reproduction for Hypogastrura viatica when exposed to different concentrations of imidacloprid. The two last values for the higher concentrations are a prediction for the age at first reproduction since they did not reproduce until the end of the experiment. Data presented as median, quartiles, 10-90 percentiles and outliers.

A dose response curve using a three-parameter log-logistic function (LL3) model was made but the data does not meet all model assumptions (Appendix, section 8.2: Figure 8.2).

3.2.4. Body size at first reproduction

Body size of *H. viatica* at first reproduction was affected negatively by imidacloprid at 0.04 mg/kg dry bark and higher concentrations (Pairwise Wilcox test: p-value<0.001; Figure 3.7), therefore 0.04 mg/kg was found to be the LOEC for size at first reproduction. The lowest concentration of 0.01 mg/kg dry bark did not affect the body size and is therefore defined as the NOEC for this trait (Pairwise Wilcox test: p-value=0.611).

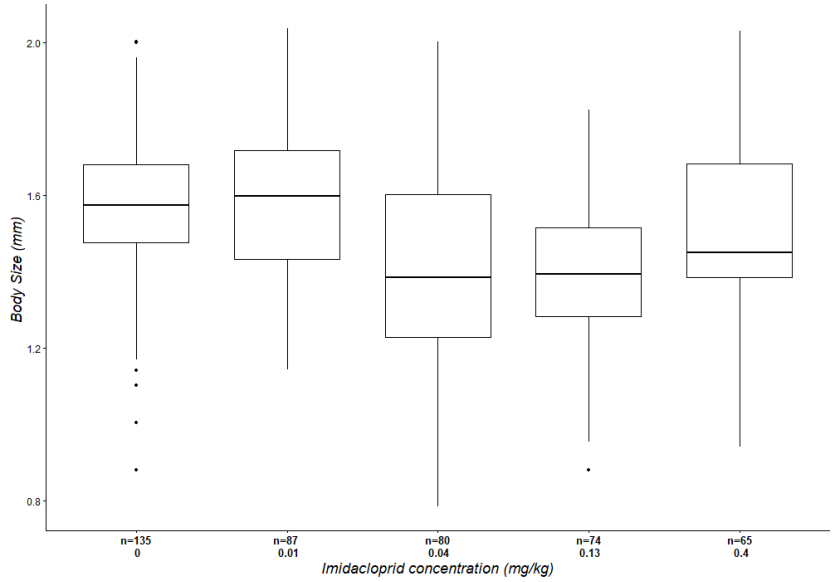

Figure 3.7 - Body size of Hypogastrura viatica when exposed to different concentrations of imidacloprid.

A dose response curve using a four-parameter log-logistic function (LL4) model was made but the data did not meet all model requirements (Appendix, section 8.2: Figure 8.3).

3.2.5. Relationship between age and body size at first reproduction

The relationship between age and body size at first reproduction within the imidacloprid treatments (or control), did not indicate a consistent trend (Figure 3.8). Body size at the age of first reproduction was dependent on imidacloprid concentration (p-value = 0.016). Since there is no consistent trend between body size and age at first reproduction, the graphic where it it possible to see the different trends split on the different concentrations was added to the appendix (Appendix, section 8.2: Figure 8.4).

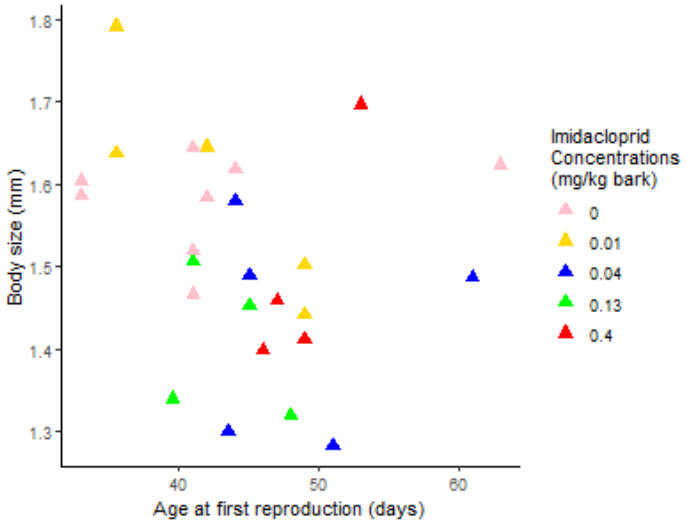

Figure 3.8 - Median body size compared with age at first reproduction for the different concentrations, for Hypogastrura viatica when exposed to different concentrations of imidacloprid.

3.2.6. Molted exuviae

The number of molted exuviae decreased for *H. viatica* exposed to the highest imidacloprid concentration of 1.2 mg/kg (Tukey HSD: p-value= 0.0508, Figure 3.9), while the lower concentrations did not affect the molting frequency (p-value> 0.05; 0.01: p-value= 0.9996892; 0.04: p-value= 0.1672; 0.13: p-value= 0.5433; 0.4: p-value= 0.8924). A linear model for molted exuviae was produced but the data does not meet all model assumptions (Appendix, section 8.2: Figure 8.6)

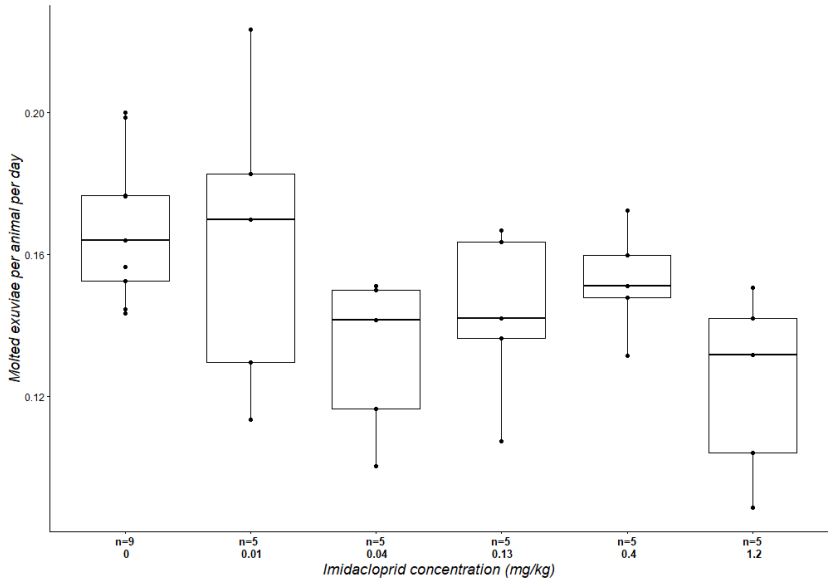

Figure 3.9 - Molted exuviae of Hypogastrura viatica when exposed to different concentrations of imidacloprid.

3.2.7. Relationship between molted exuviae and body size

The relationship between molted exuviae and body size was positive and independent for all concentrations (Figure 3.10) (slope= 3.43; p-value= 0.0001). The different concentrations were collapsed together (Appendix, section 8.2: Figure 8.7), so the interaction was not significant and body size at first reproduction and molted exuviae are not dependent on dose (p-value = 0.7).

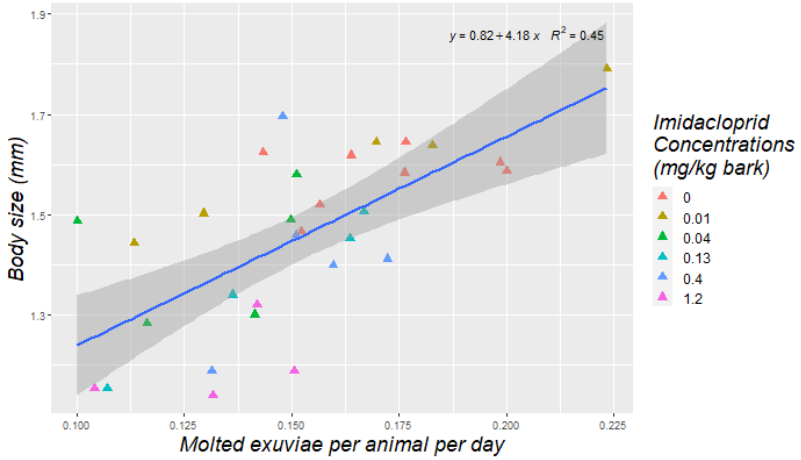

Figure 3.10 - Molted exuviae per animal per day compared with median body size (mm) for Hypogastrura viatica, when exposed to different concentrations of imidacloprid.

4. Discussion

Studying mortality is important to assess a reduction in the abundance of organisms when exposed to any contaminant (Alves *et al.*, 2014), but sub-lethal endpoints like reproduction, body growth or molted exuviae are characteristics that are easily affected when springtails are under stress (Malmström, 2012). Sub-lethal effects such as reduced reproduction, growth, and survival, ultimately cause declines in population growth (Crayton *et al.*, 2020). For widely used pesticides such as imidacloprid, although now banned, it is important to assess its toxicity in the laboratory using field relevant concentrations and soil species. In standardized toxicity tests, springtails are exposed through the ambient soil environment (OECD, 2009). Dietary exposure of contaminants to soil organisms is rarely used. For imidacloprid, dietary exposure of arthropods has been used in few studies (Table 4.1). For important soil microarthropods such as springtails, it is important to develop methods to assess the effect of contaminants on their life history traits through dietary exposure. Two springtail species, *Hypogastrura viatica* and *Folosmia quadrioculata* with differing physiology and habitat preference were used for this method development. Furthermore, with the method established, sublethal concentrations of imidacloprid on *H. viatica* was derived based on life history trait response.

Table 4.1 - Literature list of published studies relevant for this thesis, with similar experimental design to obtain information about the species of springtails used during the experiment, Folsomia quadrioculata and Hypogastrura viatica. And obtain information about imidacloprid or about the various endpoints and how these are affected when in contact with a contaminant.

Published study	Model organism	Contaminant	Type of exposure
Al-Badran *et al.*, 2019	Brown shrimp	Imidacloprid	Water
Alves *et al.*, 2014	*Folsomia candida*	6 types of pesticides	Soil
Anderson & Harmon-Threatt, 2019	Honeybee	Imidacloprid	Soil
Crayton 2020	Salamanders	Imidacloprid	Water/soil
Dai *et al.*, 2020	*Folsomia candida*	Lead	Soil
de Lima e Silva *et al.*, 2021	*Folsomia candida*	Thiacloprid; Imidacloprid	Soil
Fountain & Hopkin, 2001	*Folsomia candida*	4 types of metal	Food
Jensen *et al.*, 2006	*Hypogastrura viatica*	Nitrogen	Food
Joosse & Buker, 1979	*Orchesella cincta*	Lead	Food
Kristiansen *et al.*, 2021	*Hypogastrura viatica*	Imidacloprid	Food
Schnug, Jensen, *et al.*, 2014	*F. candida; F. fimetaria*; Earthworms	3 biocides	Soil
Sengupta *et al.*, 2020	*Folsomia quadrioculata*	Imidacloprid	Food
Szabo *et al.*, 2020	*Folsomia candida*	Trebon	Soil
Tourinho *et al.*, 2015	Isopods	$AgNO_3$	Food
van Gestel *et al.*, 2017	*Folsomia candida*	Imidacloprid	Soil

4.1.Method Development for Dietary Exposure

4.1.1. Spiking bark method

In ecotoxicological studies assessing dietary exposure to soil organisms, food is usually spiked by soaking or moistening methods (Fountain & Hopkin, 2001; Sengupta *et al.*, 2020; Tourinho *et al.*, 2015). To our knowledge there are no studies comparing the methods, and are applied on different materials, e.g., soil or leaf. In this experiment using the tree bark as food for the springtails, we demonstrated that the moistened spike method was most suitable. It was the most suitable because the measured concentrations in the food were close to the nominal unlike the soaked method. The soaked

spiking method resulted in relatively higher measured concentrations than nominal. This was consistent with Sengupta *et al*. (2020) where the authors also reported a relatively higher measured concentrations of imidacloprid in the food using the soaked spiking method. Kristiansen *et al*. (2021) adopted a similar soaked spiking method such as reported by Sengupta *et al* (2020) where Cyanobacteria were scraped from the bark, soaked in an imidacloprid solution, and then presented on a filter. However, the dietary method in Kristiansen *et al.* (2021), the scraped bark, was not an optimal feed for the species since the reproduction in control animals were halted, unlike our experiment where reproduction was observed throughout the duration of the experiment. Fountain & Hopkin (2001) also used the soaking method where yeast was soaked with four different types of metals. They measured the final concentrations of feed and showed a reduced reproduction. Tourinho *et al.* (2015) used the moistened method exposing isopods to silver nitrate ($AgNO_3$) through their food. The authors found that when exposed to contaminants throughout the feed the animals tend to be more affected than when exposed through the soil.

4.1.2. Comparison of effects on the survival and reproduction between species

When exposed to the same contaminant different species of springtails tend to have different sensitivity to toxic stress (de Lima e Silva *et al.,* 2021). This can be due to variation in reproduction method, life history traits, exposure route or different habitats (de Lima e Silva *et al.,* 2021). The results from this study indicated that *H. viatica* is more sensitive than *F. quadrioculata* for egg production. *H. viatica* being a surface-dwelling species and *F. quadrioculata* a soil litter-dwelling species, they experience different type of stressors in their natural environment. On the soil surface, *H. viatica* experience rapid changes in temperature, weather, rainfall, and wind, compared to the litter-dwelling *F. quadriculata*, which is hidden in the soil and more protected from such external factors. On the other hand, *H. viatica* is more mobile than *F. quadriculata*, moving and migrating, when in stress, in search of better-quality food and avoiding contaminated food (Jensen *et al.*, 2006). Kristiansen *et al.* (2021) and Sengupta *et al.* (2020) studied these two different species and were able to see different effects when exposed to imidacloprid between both species. While EC_{50} for *F. quadrioqulata* was considered low by the author, EC_{50} for *H. viatica* was not calculated due to limited reproduction during the experiment. When compared to *Folsomia candida*, *F. quadrioculata* is less sensitive since it had an EC_{50} higher than the ones reported in several studies exposing *F. candida* through soil (Alves *et al.,* 2014; van Gestel *et al.,* 2017). And *H. viatica* is considered more sensitive than *F. candida* when comparing EC_{50}. In our study, the low egg production in *H. viatica* could also be due to reduced growth caused by imidacloprid, as the body size was smaller than the control at the end of the experiment. A similar result of reduced growth by toxic stress has been seen for other springtails and other toxicants (Fountain & Hopkin, 2001). Therefore, it is likely that *H. viatica* had not reached its optimal body size for reproduction, which thus caused delayed maturity, and therefore reduced egg production at the time of termination. With this, it is not possible to assess *apriori* which species is intrinsically more sensitive than the other, since when subjected to different types of stress, the same species has different responses.

4.2. Concentration-Response Experiment

Concentration-response curves were attempted for all endpoints to calculate LC_x and EC_x. But since the data did not fit any common dose-response models, LC_x or EC_x values could not be estimated. The dose descriptors NOEC and LOEC were therefore applied in this study as commonly used in similar

studies (van Gestel *et al.,* 2017; Alves *et al.,* 2014). However, caution is needed when interpreting these descriptors (Warne & van Dam, 2008), as the concept of NOEC and LOEC is driven by the selected exposure concentrations used during the experiment. This means that the same experimental design can result in different NOEC and LOEC if different exposure concentrations have been applied (Chapman, 1995). On the other hand, EC_x or LC_x are interpolation values obtained manually or through a model function where it predicts the effects of a contaminant to a certain concentration (Forfait-Dubuc *et al.,* 2012). The NOEC and LOEC are calculated based on the highest concentration without a significant difference to the control (NOEC) or the lowest concentration with a significant difference to the control (LOEC) (Chapman *et al.,* 1996). Initially NOEC and LOEC were used to compare the effect of different contaminants to an organism, so it is advised to use methods that study the hypothesis where there is no difference between treatments (Laskowski, 1995), like an Analysis of variance for multiple comparisons (ANOVA). Nowadays, we use NOEC and LOEC to calculate differences within the same contaminant.

4.2.1. Field relevant concentration of imidacloprid and its effect

The effects from exposure to different concentrations of imidacloprid in *H. viatica* found in this study were similar to a previous study exposing the same species to imidacloprid through soil or food (Kristiansen *et al.,* 2021), where lower concentrations (0.001 and 0.09 mg/kg dry soil) had sub-lethal effects while the higher concentrations (0.74 mg/kg dry soil) had a lethal effect. However, all the imidacloprid concentrations used in this study caused sub-lethal effects except the highest one (1.2 mg/kg dry bark), demonstrating that the chosen concentration range was appropriate for studying sub-lethal effects in *H. viatica*. The sub-lethal effects found in this study provide important knowledge in soil ecotoxicology as the applied concentrations are within the concentration range found in agricultural fields (Anderson & Harmon-Threatt, 2019). Assuming that this study can be compared with the ones using soil as a route of exposure, the highest concentration used in this study is 1.2 mg/kg bark, while the highest concentration reported in agricultural field was 0.65 mg/kg soil (Bonmatin *et al.,* 2005). Our highest concentration is just a factor 2 higher than 0.65 mg/kg soil not showing a big difference. The remaining lower concentrations are within the field relevant range between 0.012 to 0.018 mg/kg soil (Anderson & Harmon-Threatt, 2019) and are illustrative of potential responses in the field agricultural soils. Likely effects of imidacloprid on springtails in nature are thus sub-lethal, as seen in this study. But when in the field animals are often subjected to a mixture of several different contaminants at once, and not just one contaminant at a time. Schnug *et al.* (2014) showed that different biocides can play an augmenting role in non-target species.

4.2.2. Age at first reproduction

In the two highest sub-lethal concentrations of this study (0.13 and 0.40 mg/kg), only four out of five boxes reproduced before the termination of the experiment. The lack of reproduction means that the reproductive age was delayed for these imidacloprid exposed animals. It is therefore likely that imidacloprid affects the age at first reproduction in *H. viatica*. Similar concentrations of imidacloprid cause a negative effect on egg production in *F. candida* (van Gestel *et al.,* 2017) and *F. quadrioculata* (Sengupta *et al.,* 2020). The age at first reproduction was greater for the animals exposed to 0.04 mg/kg imidacloprid compared to animals in the control, while for the animals from the remaining treatments, there were no differences from the control. However, in these studies, it is unsure whether maturity affected number of eggs produced, as this endpoint was not included in the mentioned

studies. Furthermore, the effect of imidacloprid at high exposure concentrations might be masked by high variation within each box, as age at first reproduction is determined when the first egg is found. Age at first reproduction is therefore determined when at least one female has reached maturity, while the remaining springtails may not yet be mature. Springtails must reach a certain size to physiologically be capable to produce eggs, and a high variation within the growth rate causes a need for a large sample size.

Similar studies exposed 10 days old juveniles, or adults (Sengupta *et al.*, 2020; van Gestel *et al.*, 2017), which are likely less sensitive to contaminants compared to hatchlings (< 24 hours old) we used. It is therefore likely that the hatchlings from this study were more sensitive to effects from imidacloprid contamination. Another reason for the sensitivity differences could be due to different endpoints assessed. In van Gestel *et al.* (2017) the NOEC was 0.1 mg/kg dry soil while in this study our NOEC was 0.01 mg/kg dry bark. Our NOEC value, which was 10 times lower, was estimated based on effect on body size, while van Gestel *et al.* (2017) estimated the NOEC based on recruitment from soil exposure. Moreover, the exposure routes and exposure medium used in both studies differ, and this could also be responsible for the effect we started to see at a lower concentration in *H. viatica* compared to *F. candida*. Invariably, our study showed that reproduction for *H. viatica* may be more affected by imidacloprid than *Folsomia* species consistent with the results found during method development, where *H. viatica* was more sensitive than *F. quadrioculata* in terms of egg production.

When in a contaminated site, springtails tend to reduce their reproduction in order to budget their energy for body growth (Szabo *et al.*, 2020). This reaction is a defensive mechanism that leads to a major consumption of food and a major energy uptake to grow to a favorable size (Ernsting *et al.*, 1993). A delayed reproduction leads to a lower egg production and thus reduced recruitment. Therefore, the potential delayed maturity is likely linked to body size. It is important to continue to assess recruitment since a lower egg production and a higher mortality can lead to a decreased population growth, as shown for insecticide Trebon (Szabo *et al.*, 2020) where the F3 generation for springtails was extinct. In van Gestel *et al.* (2017), the other generations showed less juveniles in each jar when exposed to imidacloprid.

4.2.3. Body size at first reproduction

Imidacloprid negatively affected the growth rate of *H. viatica*, likely because the animals spend energy on handling toxic stress, and thus less energy is available for growing. Growth rate is linked to maturity (Fountain & Hopkin, 2001), and this could explain why some boxes did not reproduce for the duration of the experiment, because they had not grown large enough. The lowest concentration, 0.013 mg/kg, was determined as the NOEC, while 0.043 mg/kg was LOEC for body size at first reproduction, indicating a high resolution in sub-lethal effects to imidacloprid.

Imidacloprid is shown to affect growth of brown shrimp, *Farfantepenaeus aztecus*, an aquatic arthropod (Al-Badran *et al.*, 2019), in which the authors suggested that imidacloprid will affect the metabolism and organisms' capacity to detoxify the contaminant leading to reduced growth. Similar effects have been observed in *F. candida* exposed to lead, causing negative effects on different endpoints, especially on growth (Dai *et al.*, 2020). There is still a knowledge gap in how sub-lethal toxic stress affects growth, important when extrapolating to population effects.

4.2.4. Molting frequency

In this experiment with juveniles, an important endpoint explaining growth and thus reproduction is the frequency of molting. This process is linked to growth, springtails need to molt their exuviae in order to grow (Dai *et al.*, 2020). The inability to molt can delay maturity, as well as reduce their excretion rate, which might impact their health. Springtails renovate their epithelium as an excretion mechanism during molting (Joosse & Buker, 1979). With exposure to higher concentrations of imidacloprid, springtails molt their exuviae with lower frequency, thus their ability to shed the exuviae is reduced. As molting is a potential route of elimination it is therefore possible that this might lead to accumulated concentrations. Imidacloprid slows down the animals´ activity in terms of walking and jumping (personal experience), almost to the point where they get motionless, disrupting and reducing molting. Kristiansen *et al.* (2021) reported that *H. viatica* exposed to imidacloprid died while molting because molting consumes energy and reduces the mobility of the animals which are under toxic stress.

4.2.5. Relationship between age at first reproduction and body size

Against all expectations, there were no relationships between age at first reproduction and body size between treatments, suggesting that there is no optimal size for first reproduction in *H. viatica*. When the relationship between the endpoints is positive, body size would be larger with greater age at first reproduction as seen in the controls, and if the relation was negative, body size would be smaller, which is practically impossible. However, the relationships between age and body size at maturity were different between the treatments. Likely, this is driven by high variation within each experimental box, where out of 20 animals, few animals likely have grown to reproductive size, although others might yet still be at immature growth stages.
Springtails need to grow to a specific size to reproduce (Ernsting *et al.*, 1993), in contrast to the findings of this thesis, this is likely explained by variation within the box. This experiment ended when one specific female reproduced, not allowing the rest of springtails to achieve the optimal size, some lower size requirement must exist.

4.2.6. Relationship between molted exuviae and body size.

A positive relationship between body size and molted exuviae was found, as expected. Molting of the exoskeleton is needed for body growth (Dai *et al.*, 2020), and although imidacloprid caused lower the number of molted exuviae and subsequently lower the body size, the relationship between the two endpoints is positive. This can thus be considered a quality assurance of the methodology of counting molted exuviae and measuring size.

5. Conclusion

Since few studies investigate the dietary exposure of soil organisms to contaminants including monitoring rarely studied traits, a better dietary exposure method had to be created as done in this study. Out of the two different applications studied, the moistened method was the most accurate to expose the test species to the desired concentration of the contaminant. Egg production of *Hypogastrura viatica* was more sensitive than *Folsomia quadrioculata* due to their adaptation to different habitats. *H.viatica* is a species that when in the presence of contaminated food is used to migrate in search of better quality food and *F. quadrioculata* is a species that is forced to deal with the changes in their habitat. Therefore, differences in intrinsic sensitivity could have played a role in the observed differences.

The concentrations chosen for the second study were considered sub-lethal apart from the highest concentration, which was lethal. The applied concentrations were selected because they can be found in farmlands and are field relevant concentrations. On exposure to imidacloprid, *H. viatica* gave preference to surviving because it reached maturity at a late age. Imidacloprid also reduced molting frequency, and thus reduced its body size since they spend more energy surviving the toxic stress as a rather than for their growth. These endpoints are important for population development, and the identified negative effects observed in this study may therefore cause population-level effects.

6. Future perspectives

A further study of this insecticide remains important as it has been banned in the EU but continues to be used to a large extent outside of Europe. And, even in places where its use is prohibited, given its potential high half-life, it is still present in the soil. When analyzing the presence of this insecticide in the field, it is important to bear in mind that this is not the only one to be used in agriculture. Even being present in the soil from past applications, it can mix with other contaminants leading to mixture effects. These effects must be studied and analyzed in future experiments, as they may have additive or synergistic effect on non-target species.

For future investigations, it is important to consider that with increasing concentrations of imidacloprid, the age at which springtails reproduce increases, so it is necessary that the same experiments have a longer period. It is important to extend the experiments in order to analyze the reproduction and eggs laid. Extending the experiment is important so as to be able to monitor the bath of eggs and their quality, since imidacloprid may affect the eggs viability and consequently population growth.

There is a knowledge gap when talking about toxic stress and how this affects growth or even molted exuviae, as these endpoints might have population effects in nature. It is important in future studies to look at these different endpoints, with this study it is possible to monitor different species without ending the experiment.

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8. Appendices

8.1. Dietary exposure

Table 8.1 - Results of the Tukey HSD for Hypogastrura viatica, estimating the differences in egg production between the different treatments. The data with a significant p-value is underline in yellow. DB – Dry bark; SB – Soaked bark; MB – Moistened bark; SSB – Soaked spiked bark; MSB – Moistened spiked bark.

	diff	lwr	upr	p adj
SB-DB	0.5013235	-0.5529434	1.55559033	0.5402636
MB-DB	-0.5346006	-1.5888675	0.51966625	0.4879866
MSB-DB	-1.4672081	-2.5214749	-0.41294119	0.0053182
MB-SB	-1.0359241	-2.0901909	0.01834278	0.0549894
MSB-SB	-1.9685315	-3.0227984	-0.91426466	0.0003480
MSB-MB	-0.9326074	-1.9868743	0.12165943	0.0927154

Table 8.2 - Results of the Tukey HSD for Folsomia quadrioculata, estimating the differences in body size between the different treatments. The data with a significant p-value is underline in yellow. DB – Dry bark; SB – Soaked bark; MB – Moistened bark; SSB – Soaked spiked bark; MSB – Moistened spiked bark.

	diff	lwr	upr	p adj
MB-DB	0.02727778	-0.02268268	0.07723824	0.4934666
MSB-DB	-0.09035948	-0.13869035	-0.04202860	0.0000132
SB-DB	0.01302972	-0.03262592	0.05868535	0.8818641
MSB-MB	-0.11763725	-0.16826123	-0.06701328	0.0000000
SB-MB	-0.01424806	-0.06232457	0.03382845	0.8697293
SB-MSB	0.10338919	0.05700841	0.14976997	0.0000001

Table 8.3 - Results of the Tukey HSD for Hypogastrura viatica, estimating the differences in body size between the different treatments. The data with a significant p-value is underline in yellow. DB – Dry bark; SB – Soaked bark; MB – Moistened bark; SSB – Soaked spiked bark; MSB – Moistened spiked bark.

	diff	lwr	upr	p adj
MB-DB	-0.06925373	-0.16956857	0. 031061106	0. 2825969
MSB-DB	-0. 18321410	-0. 28734777	-0. 079080429	0. 0000496
SB-DB	0.03736771	-0. 14301266	0. 068277244	0. 7968632
MSB-MB	-0. 11396037	-0. 21809404	-0. 009826698	0. 0257249
SB-MB	0.03188602	-0. 07375893	0. 137530976	0. 8631235
SB-MSB	0. 14584639	0. 03656872	0. 255124073	0. 0036385

8.2.Dose-response Exposure

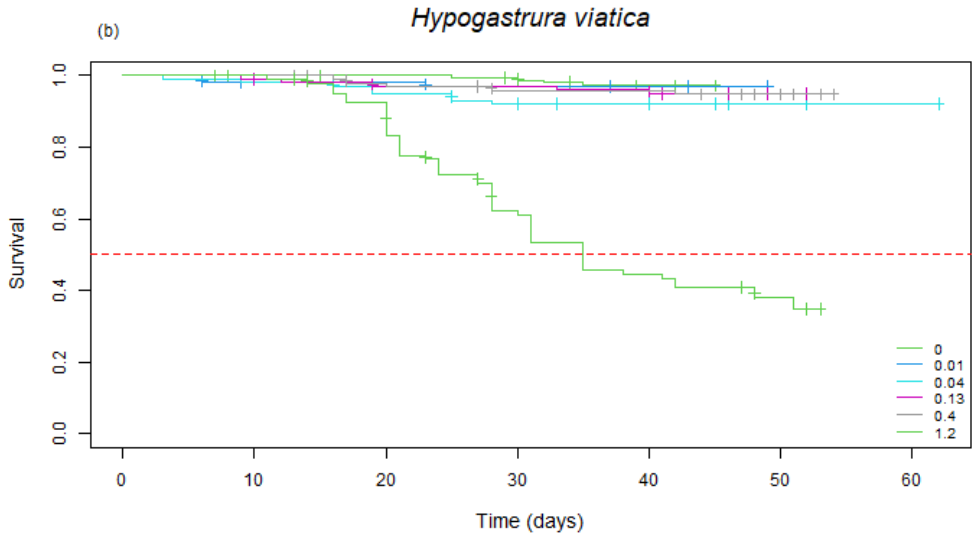

Figure 8.1 – Proportions of survival for Hypogastrura viatica exposed to different concentrations of imidacloprid. The red line marks the survival proportion of 0.5.

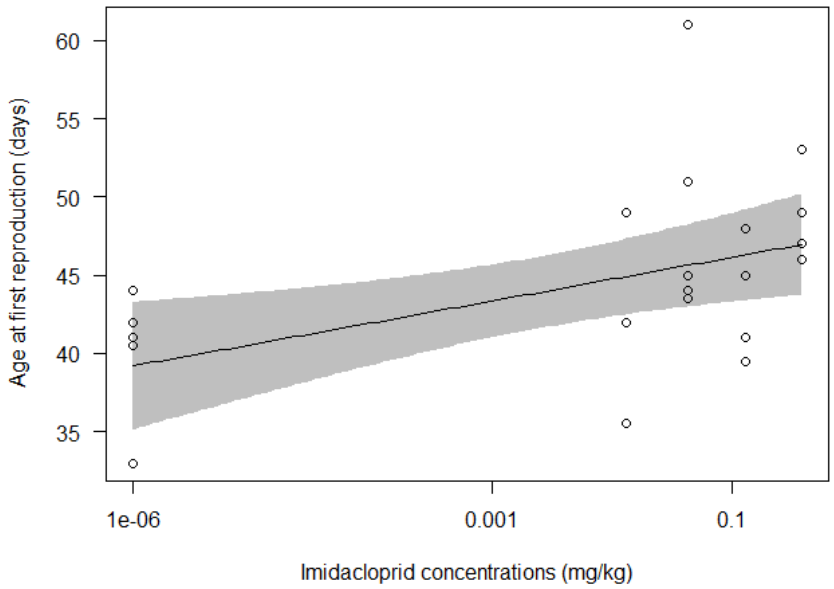

Figure 8.2 – Dose response curve using a LL3 model for age at first reproduction when exposing Hypogastrura viatica to different concentrations of imidacloprid.

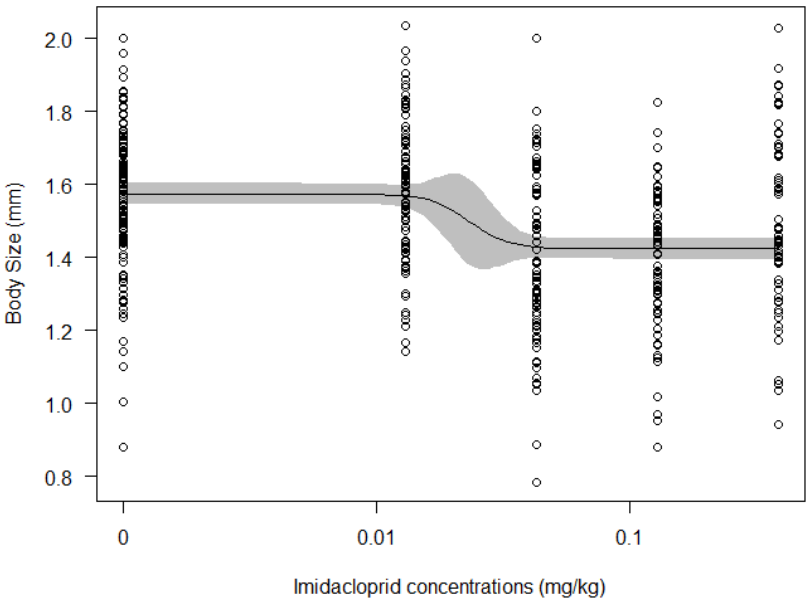

Figure 8.3 - Dose response curve using a LL4 model for body size when exposing Hypogastrura viatica to different concentrations of imidacloprid (mg/kg dry bark).

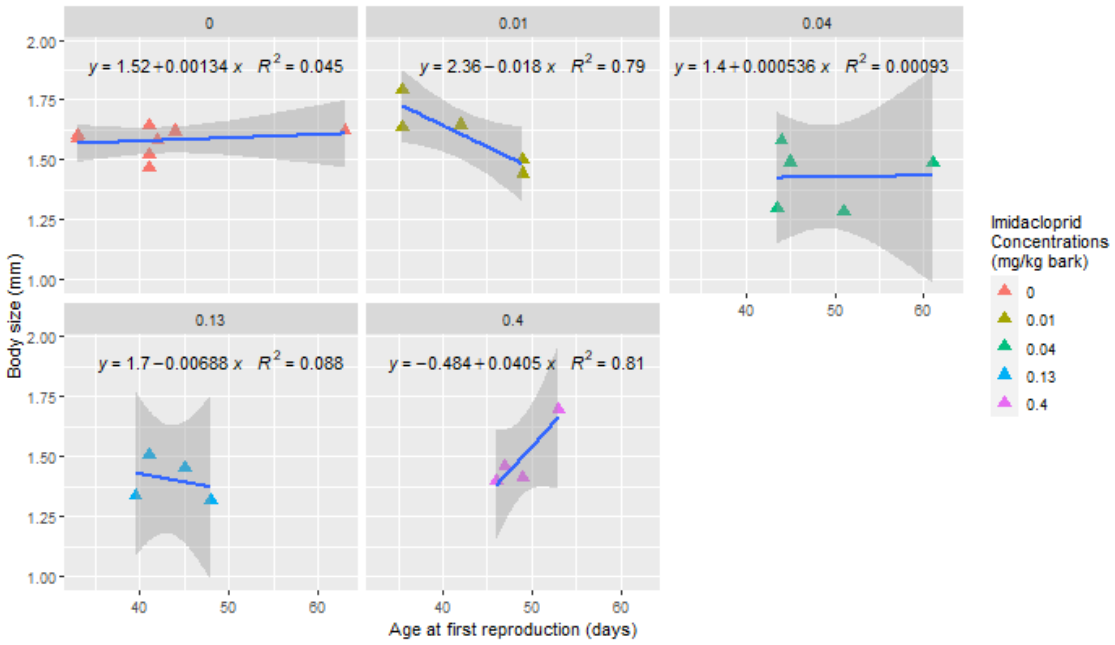

Figure 8.4 - Linear model comparing age at first reproduction and median body size at first reproduction for Hypogastrura viatica, with different trends split for each concentration.

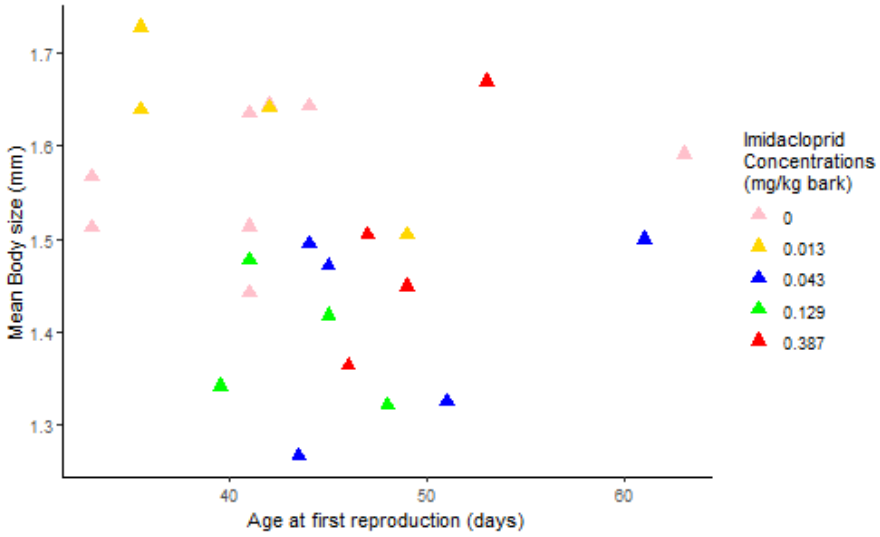

Figure 8.5- Linear model comparing the age at first reproduction (days) and mean body size (mm) when exposing Hypogastrura viatica to different concentrations of imidacloprid.

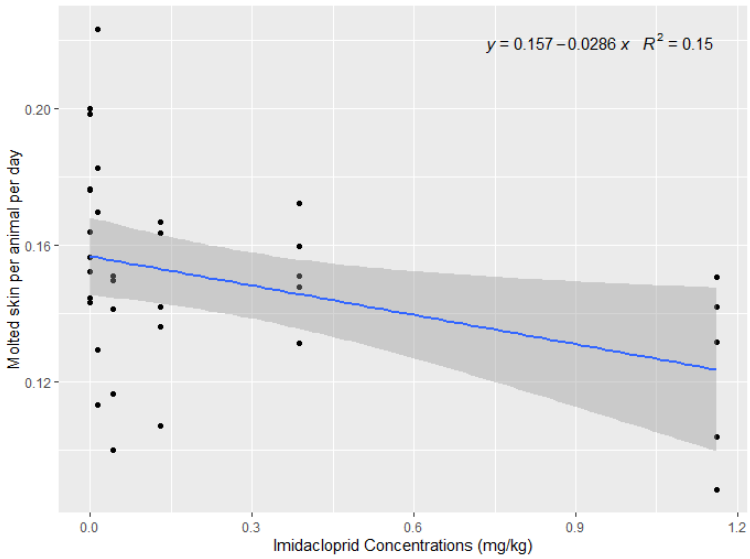

Figure 8.6 - Linear model for Hypogastrura viatica molted exuviae per animal per day through different concentrations of imidacloprid.

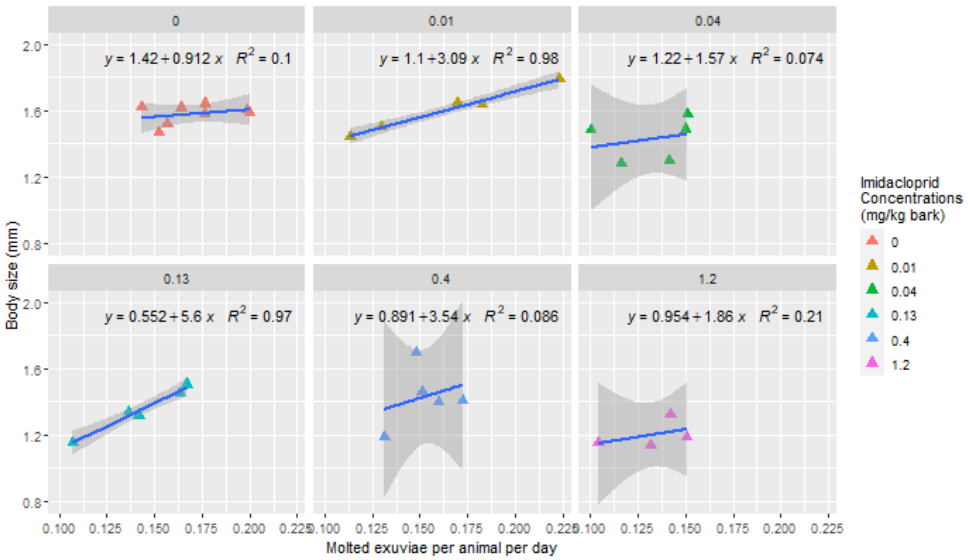

Figure 8.7 - Linear model comparing molted exuviae per animal per day and median body size for Hypogastrura viatica for each concentration separately.