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Transgenerational and multigenerational stress gene responses to the insecticide etofenprox in *Folsomia candida* (Collembola)

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ABSTRACT

Insecticide exposure may cause both transgenerational and multigenerational effects on populations, but the molecular mechanisms of these changes remain largely unclear. Many studies have focused on either transgenerational or multigenerational mechanisms but did neglect the comparative aspects. This study assessed whether the pyrethroid insecticide etofenprox (formulation Trebon® 30 EC) shows transgenerational and/or multigenerational effects on the survival and reproduction of *Folsomia candida* (Collembola). The activation of stress-related genes was studied to detect whether etofenprox modifies the expression of reproduction-associated genes in trans- and multigenerational treatments. A laboratory study was carried out for three generations with five insecticide concentrations in LUFA 2.2 soil. In the transgenerational treatment, only the parent generation (P) was exposed, but the subsequent generations were not. In the multigenerational treatment, all three generations were exposed to the insecticide in the same manner. Multigenerational exposure resulted in reduced reproduction effects over generations, suggesting that *F. candida* is capable of acclimating to enhanced concentration levels of etofenprox during prolonged exposure over multiple generations. In the transgenerational treatment, the heat shock protein 70 was up-regulated and cytochrome oxidase 6N4v1 expression down-regulated in a dose-dependent manner in the F2 generation. This finding raises the possibility of the epigenetic inheritance of insecticide impacts on parents. Furthermore, CYP6N4v1 expression was oppositely regulated in the trans- and multigenerational treatments. Our results draw attention to the differences in molecular level responses of *F. candida* to trans- and multigenerational etofenprox exposure.

1. Introduction

Many soils are receiving inputs from pesticides that can affect the populations of inhabiting species in several ways. The obligatory standard tests developed for determining pesticide effects on non-target organisms are usually short-term and cover only one generation or just a part of the organism's life cycle. These tests are not suitable to detect chronic effects that may occur following exposure over many generations (Leon Paumen et al., 2008). Repeated exposure to the same substance is a common scenario in the environment, especially in case of slowly degrading xenobiotics or the repeated use of pesticides (WHO, 2011a, 2011b). Consequently, there is a need for developing new standard methods and incorporating trans- and multigenerational tests into the risk assessment framework (Shaw et al., 2017).

Here we use the terms transgenerational and multigenerational effect according to Hanson and Skinner (2016). The term transgenerational effect is used when only the parent generation is exposed to the insecticide and offspring are living in a clean environment (without direct exposure to the insecticide). Therefore, only maternal and/or epigenetic effects could manifest in transgenerational groups. The term multigenerational effect is used when both parent and offspring generations are directly exposed to the insecticide. Therefore, it simulates repeated use of the pesticide and the accumulation of the stress effects.

A transgenerational study indicated that when *Folsomia candida* was

Abbreviations: P, parent generation; TF1, transgenerational first offspring generation; MF1, multigenerational first offspring generation; TF2, transgenerational second offspring generation; MF2, multigenerational second offspring generation; MTF2, multi-transgenerational second offspring generation; vit, vitellogenin gene; HSP70, heatshock protein 70 gene; CYP, cytochrome oxidase gene; IPNS, isopenicillin-N-synthetase gene; ABC, ATP-binding cassette transporter gene

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exposed to silver nitrate, the population could not recover in one generation kept in a clean environment. The reproduction of the first nontreated F1 generation was still lower than the control (Mendes et al., 2018). Lambda-cyhalothrin sprayed together with thiamethoxam had a transgenerational effect on F1 population emergence and sex-ratio in *Trichogramma galloi* (Hymenoptera) (Costa et al., 2014). Methoprene and teflobenzuron, two insect growth inhibitors, reduced the juvenile numbers of *F. candida* even after two generations which were not exposed (Campiche et al., 2006). Multigeneration exposure of *Enchytraeus crypticus* to copper caused an interaction between density and toxicity to offspring which was not present in the parental generation (Menezes-Oliveira et al., 2013). In a 41-generation study, *F. candida* was exposed to Cd at two concentration levels. Considerable effects were found, such as enhanced mortality in some cases, a shift in the size distribution of the collembolans and up-regulation of the metallothionein gene expression (Amorim et al., 2017). Although common risk assessment procedures currently do not address trans- and multigenerational effects, these studies indicate the need for new test methods in environmental risk assessment.

Environmental stressors, such as insecticides, can activate many genes but down-regulate others in the collembolan *F. candida* (de Boer et al., 2011). In fact, differential regulation of genes is considered as an early warning sign of homeostasis disruption due to stress, since it precedes protein translation and metabolite biosynthesis in response to a changing environment. For instance, during the biotransformation of organic compounds, Phase I involves the oxidation or the cleavage of simpler bonds, which is facilitated by monooxygenase-like cytochrome oxidases (Cytochrome P450s, CYPs) (Guengerich, 2001). This explains why CYPs are up-regulated by xenobiotics like phenanthrene (Holmstrup et al., 2014; Nota et al., 2009). The resulting metabolites often generate reactive oxygen species (ROS), which can activate antioxidant systems (Nota et al., 2009). Consequently, Glutathione-S-Transferase expression is activated to facilitate glutathione biosynthesis that will chelate the reactive metabolites so that they cannot harm cell integrity (Phase II). Finally, metabolite-glutathione complexes are excreted by ATP-binding cassette transporters (ABC transporters) (Sillapawattana and Schäffer, 2017; van Straalen and Roelofs, 2012). Heatshock proteins (HSPs) are part of the general stress-response to xenobiotics and other stressors (Sørensen et al., 2003). Increased expression of HSPs seems to extend lifespan of *Drosophila melanogaster* (Sarup et al., 2014). A lower expression of vitellogenin and vitellogenin-like protein could be indicative of adverse effects on reproduction, as a sublethal sulfoxaflor concentration decreased vitellogenin expression and net reproductive rate in *Apolygus lucorum* (Miridae) (Zhen et al., 2018).

Trebon® 30 EC is a pyrethroid insecticide formulation, containing the active ingredient etofenprox. This pesticide is recommended against insect pests affecting different vegetables, cereals, fruits, and flowers. Etofenprox is used all over the world (Boina and Bloomquist, 2015; Martin et al., 2002; Vatandoost et al., 2004), but it is especially important in areas with mosquito vector-borne diseases where it is applied repeatedly throughout the year (WHO, 2011b, 2011a). In East-Asia, etofenprox is effective against rice planthoppers, cabbage caterpillars, and the cotton bollworm (Zhang et al., 2013). According to the instructions for use the recommended field dosage of the insecticide ranges from 0.15 to 0.6 L Trebon® 30 EC/ha, leading to an estimated concentration of approximately 0.3–1 mg/kg etofenprox in the top 1 cm of the soil.

Data on the effects of etofenprox on non-target soil arthropods are scarce, but multigenerational experiments in order to examine etofenprox effects on the life-history of *F. candida* were performed (Szabó et al., 2018; Szabó and Bakonyi, 2017).

F. candida Willem, 1902 (Collembola, Isotomidae) is a widely used model animal in soil ecotoxicology. This species is distributed all over the world in organic matter-rich soils. *F. candida* is easy to rear in the laboratory because of its parthenogenetic way of reproduction (Fountain and Hopkin, 2005). *F. candida* has a role in the humification of organic matter and the regulation of the soil microbial community (Hopkin, 1997). This species has a short generation time (about one month) which makes it suitable for multiple generation testing (Wiles and Krogh, 1998). Although *F. candida* does not seem to have a methylation pattern (Noordhoek et al., 2018), transcriptome analysis could be indicative of the epigenetic status of the animals. Moreover, the genome of this species is reasonably well known (Faddeeva-Vakhrusheva et al., 2017).

The primary objective of this paper is to assess whether etofenprox, in the formulation Trebon® 30 EC, has transgenerational and/or multigenerational effects on (i) the survival and/or reproduction, and (ii) gene expression of *F. candida*. It also aims at unravelling (iii) which stress genes are activated and (iv) whether reproduction-associated gene expression is modified by exposure to etofenprox. As a starting point, we selected ABC-transporter, isopenicillin-N-synthetase, cytochrome P450 monooxygenases (CYP6N3v2, CYP6N4v1), and heatshock protein 70 genes, that were shown to respond to organic compound/pesticide toxicity based on previous research.

2. Materials and methods

2.1. Test organism

The stock culture of *Folsomia candida* Willem (1902), Berlin strain, at the Department of Ecological Science at the Vrije Universiteit Amsterdam was kept in a climate room at 16 ± 2 °C, 12 h light/12 h darkness and 75% relative humidity. Cultures were kept in plastic boxes with a moist layer of plaster of Paris and active charcoal (10:1 W/W). Synchronization was done in a climate room at 20 ± 2 °C using similar plastic boxes. The experiments were performed in a climate room at 20 ± 2 °C. The animals were fed with baker's yeast (Instant yeast from Algist Bruggeman N.V, Ghent, Belgium) *ad libitum* in the stock culture and during the experiment.

2.2. Test soil and chemicals

All tests were performed in LUFA 2.2 natural soil (LUFA Speyer, Germany), a loamy sand with 1.59 ± 0.13% organic carbon, pH_{CaCl2} of 5.4 ± 0.2, and water holding capacity (WHC) of 43.5 ± 2.8%, or on plaster of Paris with active charcoal. Trebon® 30 EC (Mitsui Chemicals) is a liquid insecticide, with the active substance etofenprox (pyrethroid). Etofenprox is very lipophilic, but the formulation can be dispersed in water. Based on the results of earlier tests, the following concentrations were chosen: 107, 179, 299 and 500 mg active substance/kg dry soil and a control receiving demineralized water. The soil was spiked half a day before use. Every batch of soil was mixed thoroughly with the proper solution to reach a homogenous state and left in a fume hood to allow evaporation of the formulating agents. Also, fume hood pre-treatment was necessary to mimic field-conditions, where the formulation additive naphthalene has a chance to evaporate. Before use, the water content of the soil was corrected and adjusted to 22% (corresponding with 50% of its WHC). Thirty gram moist soil was placed in each jar, with ten replicates in the control and five for each test concentration.

2.3. Treatment groups and experimental design

The experimental design of the trans- and multigenerational exposures is shown in Fig. 1.

The OECD 232 *F. candida* reproduction test (OECD, 2009) was used as the basic design for the ecotoxicological part of the exposure of every generation. The animals were 10–12 days old at the start of each generation. The juveniles produced by the P generation were harvested using a sieve after flooding the test jars to make all animals floating. In the second generation (F1), a control (C), a transgenerational (TF1) and

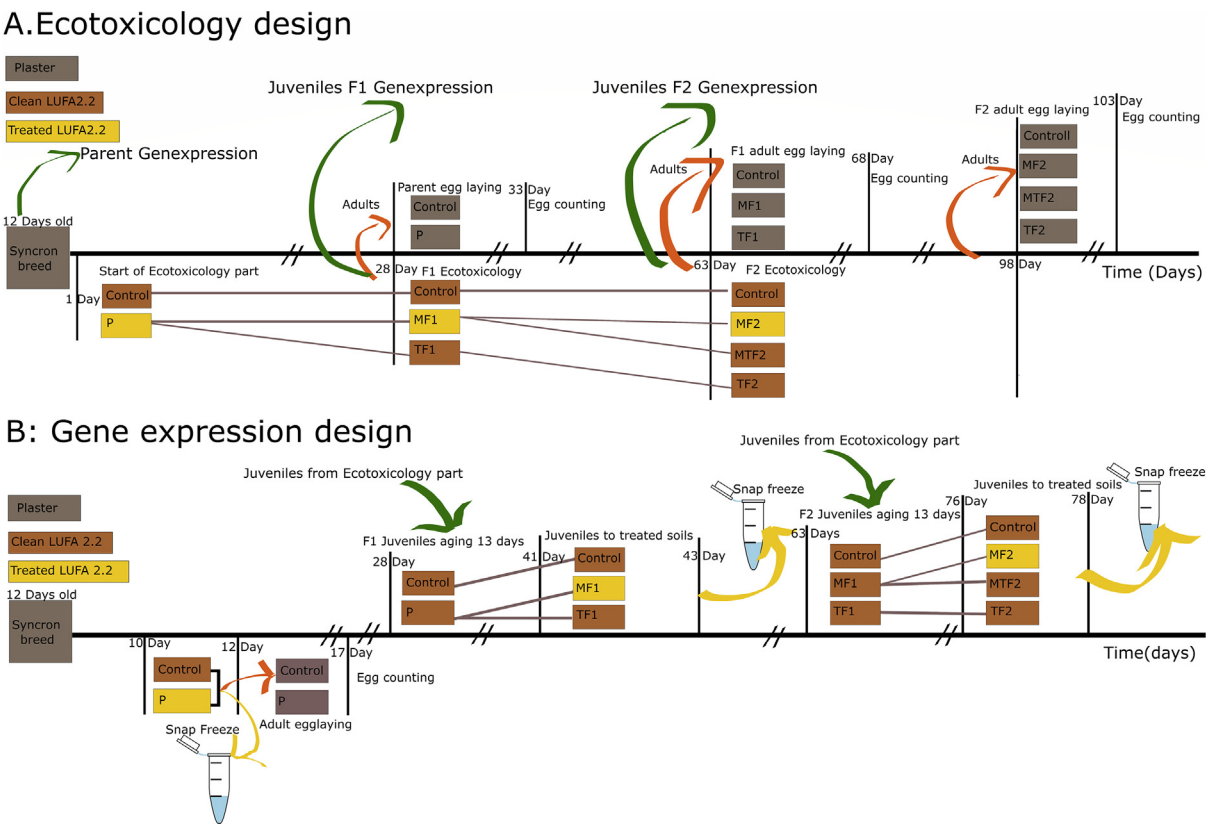

Fig. 1. The experimental design chosen in this study to assess the multigenerational and transgenerational effects of etofenprox on *Folsomia candida*. A: Ecotoxicology: the design included a sequence of OECD toxicity, egg laying, and gene expression experiments. B: Gene expression. The colour of the boxes signifies the medium the collembolans were kept in during the different phases of the experiment (grey-plaster, brown-clean LUFA soil, yellow-treated LUFA soil). The horizontal line shows the timeline, which is not proportional (shown with quotation mark). The vertical black line which goes through the timeline separates the generations. Treatment codes: P: parents, TF1: first generation transgeneration treatment, MF1: first generation multigeneration treatment, TF2: second generation transgeneration treatment, MF2: second generation multigeneration treatment, MTF2: second generation multi-transgeneration treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

a multigenerational (MF1) treatment were set-up. The multigenerational group of Collembola was transferred into treated soil, while the transgenerational group was transferred into clean soil. The animals from treatments were randomly assigned to the transgenerational and multigenerational test jars, while control animals were transferred to jars with control soil. Juveniles from the F1 control group were transferred to clean soil to form the control group of the third generation (F2). The transgenerational group was taken from F1 transgenerational juveniles of the given concentration (TF2). Moreover, the F1 multigenerational group was divided into two more groups; a multigenerational group in treated-soil (MF2) and a multi-transgenerational group (MTF2) in non-treated, clean soil.

In the ecotoxicology part of the study (Fig. 1A), the P-generation was exposed for 28 days in the soil, then the adults and juveniles were counted. Five adults were put on plaster in a plastic box for five days to lay eggs. After removal of the adults, digital photos were taken from the spread eggs. The egg number was counted, and the egg size measured with the shortest and the longest diameter of the eggs (Image J software; Schneider et al., 2012). Ten eggs were chosen randomly for size measurement. The volume of the eggs was calculated with the prolate spheroid equation (Satterly, 1960).

For the F1 and F2 populations, the exposure period in LUFA soil was extended to 35 days to get enough 10–12 day old animals. In the ecotoxicology part of the study, the F1 and F2 treatment group were assessed in the same way as described above (adult and juvenile counting, adult egg laying and egg parameter measurement).

In the gene expression study (Fig. 1B), the P-generation animals were obtained from the same synchronized population as in the ecotoxicology part, with the exception that the animals were 22 days old. The animals were exposed to etofenprox for two days in LUFA 2.2 soil, spiked with the same concentrations as described above. Each jar contained 30 g moist soil, using 50 animals to determine gene expression and five animals testing reproduction-related genes. After two days, the animals were extracted from the soil by flooding; five animals were transferred to a plastic box with a plaster bottom to lay eggs for five days. After removal of the adults, digital photos were taken from the spread eggs. The egg parameters were measured in the same manner as in the ecotoxicology part of the study. Fifty animals were snap frozen in liquid nitrogen and stored until usage at −80 °C. In the F1 generation the juveniles were obtained from the P generation jars at the end of the 28 days ecotoxicology test. Juveniles of the F1 generation were incubated in clean soil for 13 days to reach proper size and age for determining gene expression responses. Then they were exposed for two days to the same concentration (multi- and transgenerational) as the ecotoxicology groups, using fifty animals per jar. In the F2 generation, juveniles were obtained from F1 generation jars after 35 days of the ecotoxicology test and exposed following the same procedure as the F1 generation juveniles (trans-, multi-, and multi-transgenerational treatment). After two days of exposure, animals of both the F1 and F2 generations were snap frozen, then stored at −80 °C.

2.4. RNA extraction

Total RNA was extracted from about 50 snap frozen collembolans per biological replicate. The SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA) was used for RNA extraction according

to the manual, except that the DNase incubation mix was applied for 30 min for more optimal DNA degradation. RNA concentration was measured on a NanoDrop ND-1000 spectrophotometer (Wilmington, DE, USA) and stored at −80 °C.

2.5. RNA quality check

The RNA was run on a 1% agarose gel (SphaeroQ) with ethidium bromide to check for fragmentation. Then a PCR was done on all RNA samples to check for DNA contamination. We used general house-hold gene DNA primers, eukaryotic transcription initiation factor IA (Etif) primers and the GO-taq PCR protocol of Promega (56 °C for 30 cycles). The PCR product was run on 1.5% agarose gel with ethidium bromide to check DNA content visually.

2.6. cDNA synthesis

Five µl RNA sample (mean concentration: 137.74 ng/µl) was incubated at 70 °C for 5 min, then chilled on ice. Twenty µl reverse transcription mix was added (5 µl 5× buffer, 1,5 µl 10 mM dNTP, 0,5 µl oligo dT in 1 µg/µl concentration, 1 µl MMLV reverse transcriptase (200 units), 12 µl nuclease free water) and the mixture was incubated in the thermocycler to a cycle of 5 min at 25 °C, 60 min at 42 °C and 15 min at 70 °C. The cDNA was diluted with 100 µl nuclease free water.

2.7. Gene expression analysis

Gene expression was analysed by quantitative real-time PCR (qPCR) using the following qPCR assays. Two house-keeping genes were used as reference-genes: tyrosine 3-monooxygenase (YWHAZ) and succinate dehydrogenase (SDHA) (de Boer et al., 2009). Five general stress-genes were used in the analysis: ABC-transporter (ABC), isopenicillin-N-synthetase (IPNS), two versions of cytochrome P450 monooxygenases (CYP6N3v2, CYP6N4v1), and heatshock protein 70 (HSP70). We designed three qPCR assays for reproduction-associated genes: vitellogenin protein (vit-1), vitellogenin-like protein (vit-2), and vitellogenin-receptor (vitrec). Vitellogenin and vitellogenin-like protein are both important components of the eggs. Vitellogenin-receptor not only helps the vitellogenin to be transported into the eggs but also has a role in the maturation of the ovaries (Cong et al., 2015). The genes' annotation numbers, primer sequences, and efficiencies are shown in Table 1. First, the qPCR mix was made (0.5 µl forward primer, 1.5 µl reverse primer, 7 µl nuclease free water, 10 µl sybr green from Bioline, UK) and distributed over the 96 wells of the qPCR plate, then 2 µl sample was added into the wells. Biorad CFX qPCR was used in the assay (initiation 50 °C for 10 min, 95 °C for 5 min, 30 cycles of 10 s at 95 °C and 30 s at 60 °C). Specificity of PCR products was confirmed after each amplification by analysis of the melting curve; 60–95 °C with a heating rate of 0.1 °C per second and one fluorescence measurement per second. Each run included a non-template control for each assay (de Boer et al., 2009).

2.8. Statistics

All statistical analyses were made using the R Statistical program 3.4.2 (R Core Team, 2017). The effects of the insecticide on the survival, juvenile number, egg number and RNA amount per animal (total RNA amount per Eppendorf-tube corrected with the number of animals in the tube) were analysed with a linear model, using Dunnett's test as the posthoc test. In case of the egg numbers in the F2 multi-transgenerational exposures, the effect was analysed with a piece-wise generalised linear model because the relation between data points was non-linear (MASS package from R; Venables and Ripley, 2002). The egg volume was analysed with a mixed effect linear model (nlme package from R; Pinheiro et al., 2013). The identification number of the individuals was the random subject, which showed that the eggs originated from the same dish. In order to gain normality of the data, the egg sizes were cube root transformed. The gene expression data were log transformed (log10). The effect of the insecticide concentration on the gene expression was analysed with a linear model. In the case of IPNS of the P generation, and ABC in the F2 multi-transgenerational exposures, a piece-wise generalised linear model was applied. After transformation, all data sets met the requirements of normality (Residual variances, QQ plot, Cook distance plot). The treatment groups MF1 and TF1, MF2, TF2, and MTF2 were analysed separately. No observed effect concentration (NOEC) and lowest effect concentration (LOEC) were calculated. LOEC was used in the comparison between generations and treatments. The 50% effect concentration (EC50) and 95% confidence intervals were calculated with ToxRat (ToxRat®Solutions.Gmbh, 2015). Due to the great differences between treatments, populations and generations in terms of the number of samples and other parameters (like exposure times), it was not possible to compare the data of

Table 1
Information on the qPCR assays used to assess the effect of etofenprox exposure on the gene expression of *Folsomia candida*. F: forward primer, R: reverse primer. Annotation numbers found at http://collembolomics.nl.

Function	Gene code	Annotation	F/R	Primer sequence	Efficiency
vitellogenin protein[a]	vit1	Fcan01_28655	F	GAGTTTCTGCTGACGGACACCT	100.3
			R	TGTTCAGACTCGCCACCAGAT	
Vitellogenin-like protein[a]	vit2	Fcan01_06376	F	GTTTCTGCTGACGGACACCTTC	95.3
			R	AAATGTTCAGACTCGCCACCA	
vitellogenin receptor[a]	vitrec	Fcan01_04245	F	TGTCCCGTAGGGATGTATCTTGA	85.6
			R	GATTGTGTTGTTGTACCCGATGAC	
Tyrosine 3-monooxygenase[b]	YWHAZ	Fcan01_06830	F	CCTACAAAAACGTCGTCGGTG	89.3
			R	TGTTGCTTTCGTTCGAACC	
Succinate dehydrogenase[b]	SDHA	Fcan01_08383	F	ACACTTTCCAGCAATGCAGGAG	98.6
			R	TTTTCAGCCTCAAATCGGCA	
ABC-transporter[b]	ABC	Fcan01_27073	F	GTGTGAAATCTGGCGAAAAGGT	90.6
			R	TTGAGCAGCAGAAGGCACTAATC	
cytochrome P450 monooxygenase[b]	CYP6N3v2	Fcan01_00866	F	GCGTTAAAAGCGAGGCAAGA	89.3
			R	GCGATATCCACGTTCGAATTGT	
cytochrome P450 monooxygenase[b]	CYP6N4v1	Fcan01_20588	F	TTCCATGCAAGTCATCACATCAG	106.5
			R	CGGAAACACAAAGATTCGTTCTG	
Heat Shock Protein 70[b]	HSP70	Fcan01_10021	F	TTGGTCGACGTAGCTCCACTCT	98.1
			R	TGGGCTTGTTTGCATGGAAT	
Isopenicillin-N-synthetase[b]	IPNS	Fcan01_06374	F	GACATGTCGGCAAAACTCCTTC	84.4
			R	GGGTAGCGAATAAGTCGCACTG	

[a] New assays.
[b] See specifications: Faddeeva-Vakhrusheva et al. (2017).

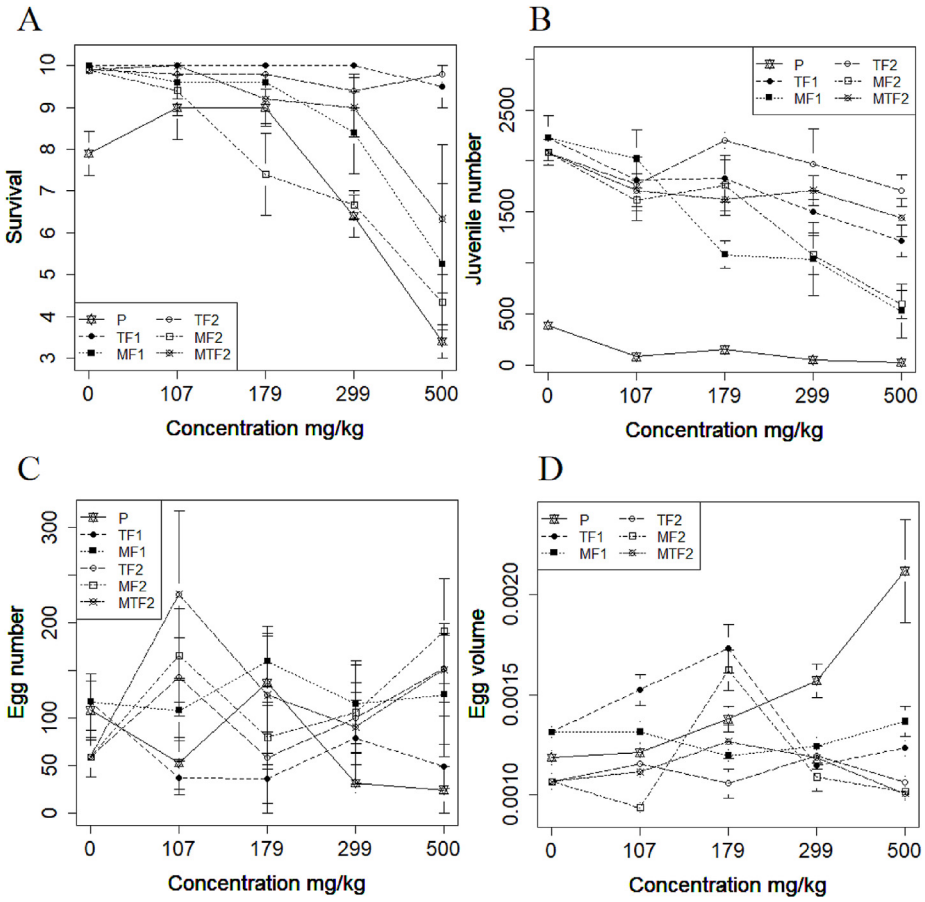

Fig. 2. Response of *Folsomia candida* to etofenprox (in the formulation Trebon® 30 EC) in LUFA 2.2 soil. A: survival, B: juvenile number, C: egg number, D: egg volume. P: parents, TF1: first generation transgeneration treatment, MF1: first generation multigeneration treatment, TF2: second generation transgeneration treatment, MF2: second generation multigeneration treatment, MTF2: second generation multi-transgeneration treatment. The symbols are the mean, the whiskers are the standard error. Please note that the juvenile number in the parent generation was lower than in the later generations due to the experimental setup (see Materials and Methods).

different generations using ANOVA or other statistical tests. The heat plots were drawn and clustered (distances of Pearson correlation) with TIGR MeV software (Sharov et al., 2003).

3. Results

3.1. Ecotoxicological effects

Test validity criteria required by ISO (1999) and OECD (2009) standard tests were almost completely fulfilled in our studies (adult mortality in controls < 20%; mean number of juveniles > 100 per jar; coefficient of variation of the juvenile number < 30%). In the P generation, mortality in the control was 21% and coefficient of variation of the juvenile number was 35.3%. The low control survival and high variation in control reproduction in the P generation were caused by one replicate having only 40% survival and 142 juveniles, which are both significant outliers (Chi-squared test $p < 0.02$). In F1 generation, variation coefficient was 31.6%, slightly higher than the OECD test validity criteria.

Fig. 2 shows the effects of etofenprox on springtail survival, reproduction and egg volume; the corresponding endpoints are depicted in Table 2 and the significance levels in Table S1. For parents, the LOECs for effects on survival and juvenile numbers were 500 and 107 mg/kg, respectively. The egg number was not significantly affected, but the egg volume increased with increasing etofenprox concentration (LOEC 299 mg/kg).

In the MF1 generation, springtail survival and juvenile number decreased with increasing etofenprox concentration (LOEC 500 and 179 mg/kg, respectively). The egg number and the egg volume were not significantly affected by the pesticide. In the TF1 generation, survival decreased slightly but not significantly with increasing etofenprox concentration. The juvenile number decreased with increasing etofenprox concentration (LOEC 500 mg/kg). The egg number was not significantly affected by the pesticide. The egg volume slightly but not significantly increased with increasing etofenprox concentration.

In the MF2 generation, survival and juvenile number decreased with increasing etofenprox concentration, LOECs were 179 and 299 mg/kg, respectively. The egg number was not significantly affected by the pesticide. LOEC for effects on egg volume was 179 mg/kg. In the TF2 generation, neither survival nor juvenile number, egg number and egg volume were affected by the pesticide.

In the MTF2 generation, survival decreased with increasing etofenprox concentration (LOEC 500 mg/kg), but the juvenile number and egg number did not. The egg volume was significantly larger at 107 mg/kg, and showed a clear hormetic effect ($p = 0.031$, $t = 2.29$).

A significant dose-related increase of springtail survival was found for the P, MF1, MF2 and MTF2 exposures. Survival was most affected in the MF2 treatment (LOEC 179 mg/kg). The juvenile number was lower in the P generation than in the other ones due to the shorter exposure period. Significant dose-related reductions of juvenile numbers were found in the P, MF1, TF1 and MF2 treatments. Effects were most pronounced for the P generation, where etofenprox effects were significant already at 107 mg/kg.

Comparing the LOEC values (Table 2), the survival of the F1 and F2 generations was higher than that of the P generation at all etofenprox concentrations (Fig. 2A). No such clear difference is visible in case of the MF1 and MF2 generations. The juvenile number was higher in the TF2 treatment than in the TF1 at higher insecticide concentrations (Fig. 2B). The LOEC for effects on juvenile numbers increased through the multigeneration treatment (P < MF1 < MF2). No clear differences in egg number were seen between any of the treatments (Fig. 2C). The egg volume showed an apparent positive dose-related response only in the P treatment (Fig. 2D).

Table 2
The NOEC, LOEC, and EC50 values for the effect of etofenprox (in the formulation Trebon® 30 EC) on the survival, reproduction and gene expression of *Folsomia candida* in LUFA 2.2 soil (in between brackets the 95% confidence interval). When the etofenprox effect was not significant, no post-hoc test was applied. The values marked with an asterisk indicate a hormetic effect. The vertical line separates the different generations. The etofenprox concentrations are in mg/kg dry soil. P: parents, TF1: first generation transgeneration treatment, MF1: first generation multigeneration treatment, TF2: second generation transgeneration treatment, MF2: second generation multigeneration treatment, MTF2: second generation multi-transgeneration treatment, n.c.: not calculable.

	P		MF1		TF1		MF2		MTF2		TF2	
	LOEC	EC50	LOEC	EC50	LOEC	EC50	LOEC	EC50	LOEC	EC50	LOEC	EC50
Survival	500	387 (326–494)	500	241 (54–1051)	> 500	–	179	413 (320–667)	500	> 500	> 500	> 500
Juvenile number	107	30 (0.003-n.c.)	179	> 500	500	> 500	299	331 (105–1010)	> 500	–	–	–
Egg number	> 500	–	> 500	–	> 500	–	> 500	–	> 500*	–	> 500	–
Egg volume	299	> 500	–	–	–	–	> 500*	–	–	–	–	–
RNA	500	507 (172–1527)	179	211 (75–594)	179	201 (74–547)	299	404 (102–1620)	–	–	–	–
vit1	–	–	299	287 (n.c.)	–	–	–	–	–	–	–	–
vit2	–	–	299	126 (n.c.)	–	–	–	–	–	–	–	–
vitrec	500	–	–	–	–	–	> 500	> 500	–	–	–	–
ABC	500	169 (n.c.)	–	–	–	–	299	115 (n.c.)	> 500*	–	–	–
CYP6N3v2	107	88 (25–317)	107	78 (40–151)	–	–	107	91 (37–225)	< 107	–	–	–
CYP6N4v1	107	76 (30–197)	107	21 (9–49)	–	–	107	89 (27–293)	< 107	> 500	500	–
HSP70	< 107	–	179	113 (33–397)	> 500*	26 (0.34–2092)	179	137 (50–375)	179	137 (134–139)	299	165 (74–364)
IPNS	107	–	< 107	–	< 107	–	< 107	–	< 107	–	< 107	–

3.2. Gene expression

In the P generation, RNA yield per animal decreased with increasing etofenprox concentration, LOEC was 500 mg/kg (Figure S1, Table 2). Only one reproduction gene, Vitrec, was significantly down-regulated upon increasing etofenprox concentrations (LOEC 500 mg/kg) (Figure S2.). Almost all stress-related genes were strongly affected by etofenprox (Figures S2-S7.). The expression of ABC and both CYP6N3v2 and CYP6N4v1 increased with increasing concentration (LOECs 500 and 107 mg/kg, respectively). The expression of HSP70 showed a slight but non-significant increasing trend with increasing exposure concentration. A non-linear dose-related response was found for IPNS expression (Figure S5.): expression was significantly decreased at the lowest concentration ($p < 0.001$, $t = -4.19$), but significantly increased with increasing concentration ($p = 0.010$, $t = 2.78$) and was significantly higher than the control at the two highest concentrations ($p < 0.001$, $t = 5.30$).

In the MF1 generation, RNA yield per animal decreased with increasing etofenprox concentration (Figure S1), LOEC was 179 mg/kg (Table 2). Vit1 and vit2 reacted with an increased expression at increasing etofenprox concentrations (LOEC 299 mg/kg), and so did the expression of both CYP6N3v2 and CYP6N4v1 (LOEC 107 mg/kg) (Fig. 3) and HSP70 (LOEC 179 mg/kg). In the case of ABC and IPNS, the expression at 500 mg/kg was an outlier, after removal there was no significant trend in the expression. For both genes expression at 500 mg/kg was significantly higher than in the control, but based on data of only one sample.

In the TF1 generation, RNA yield per animal decreased with increasing etofenprox concentration (Figure S1), LOEC was 179 mg/kg (Table 2). Only the expression of HSP70 increased with increasing etofenprox concentration (LOEC 179 mg/kg).

In the MF2 generation, RNA yield per animal decreased with increasing etofenprox concentration (Fig. S1), LOEC 299 mg/kg (Table 2 and Table S2). Vitrec was down-regulated with increasing etofenprox concentrations, but not in a dose-dependent manner. The expressions of ABC, both CYP6N3v2 and CYP6N4v1, and HSP79 (Fig. 4) increased with increasing concentration; LOECs were 299, 107 and 179 mg/kg, respectively.

In the MTF2 generation, RNA yield per animal was not affected by etofenprox concentration. ABC expression was significantly higher at 107 mg/kg than in the control (approx. 250%). CYP6N3v2 expression slightly but not significantly increased with increasing exposure concentration. CYP6N4v1 expression decreased while HSP70 expression (Fig. 4) increased with increasing concentration; LOECs were 179 mg/kg in both cases.

In the TF2 generation, RNA yield per animal was not affected by etofenprox. The expression of HSP70 (Fig. 4) increased while CYP6N4v1 expression (Fig. 3) decreased with the increasing concentration; LOEC was 299 and 500 mg/kg, respectively.

The expression results clearly show that stress-related genes showed increased responses along the multigenerational treatment (Fig. 5A). Cluster analysis of gene expression profiles indicates that transgenerational treatments resemble gene expression levels similar to control samples, while multigeneration treatments grouped with the parent generation treatments (Fig. 5B). The transgenerational treatment caused a constitutive activation of HSP70 and a decrease of the CYP6N4v1 level. The multi-transgenerational treatment responded similar to the TF2 treatment, except that MTF2 also showed activated

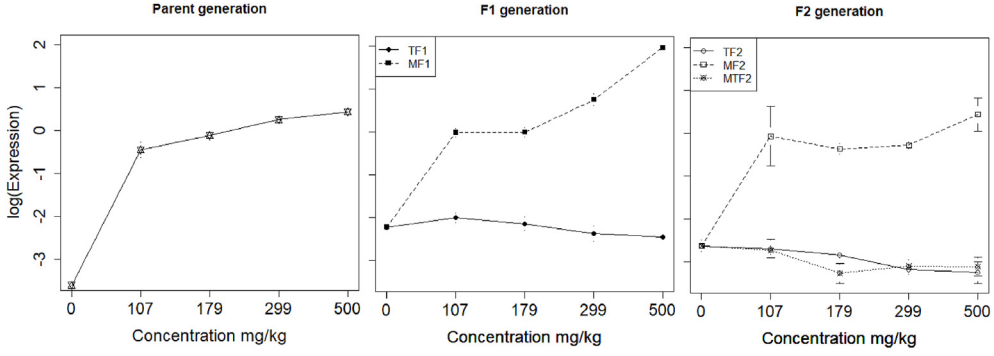

Fig. 3. Mean (± standard error) expression of the cytochrome 6N4v2 gene over different generations of *Folsomia candida* exposed to etofenprox (in the formulation Trebon® 30 EC) in LUFA 2.2 soil. P: parents, TF1: first generation transgeneration treatment, MF1: first generation multigeneration treatment, TF2: second generation transgeneration treatment, MF2: second generation multigeneration treatment, MTF2: second generation multi-transgeneration treatment.

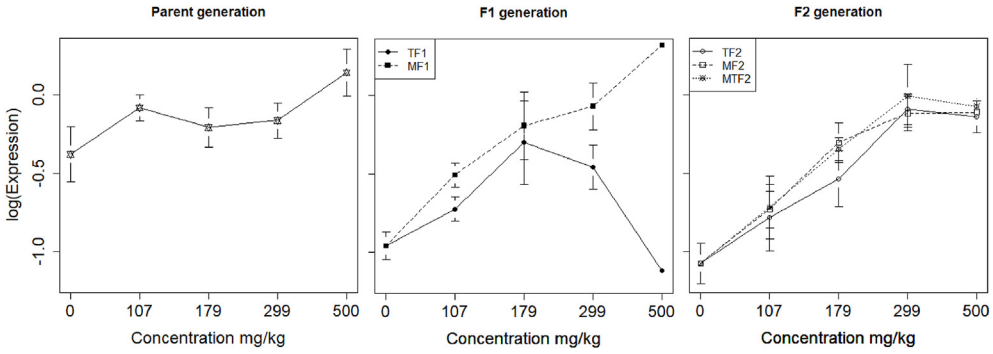

Fig. 4. Mean (± standard error) expression of the HSP70 gene over different generations of *Folsomia candida* exposed to etofenprox (in the formulation Trebon® 30 EC) in LUFA 2.2 soil. P: parents, TF1: first generation transgeneration treatment, MF1: first generation multigeneration treatment, TF2: second generation transgeneration treatment, MF2: second generation multigeneration treatment, MTF2: second generation multi-transgeneration treatment.

CYP6N3v2. During the multigenerational treatment, ABC, CYP6N3v2, CYP6N4v1, and HSP70 were markedly activated.

4. Discussion

4.1. Ecotoxicology

Etofenprox, tested in the formulation Trebon® 30 EC, exerted both transgenerational and multigenerational effects on *F. candida*. Transgenerational effects on survival and reproduction were observed only in the F1 generation. However, this effect may be due to exposure of F1 juveniles to the insecticide. Apparently, the animals were able to fully recover in a subsequent generation. Multigenerational effects became evident from a lower sensitivity of reproduction (higher juvenile numbers). The lower sensitivity could be the beginning of a resistance, or the resilience of the population against etofenprox (Trebon® 30 EC) over time.

The transgenerational effect on the survival and reproduction of *F. candida*, observed in the F1, may be explained by epigenetic imprinting or the transmission of the pesticide through egg content. Epigenetics is the study of heritable changes in gene function that occur without mutation in the DNA sequence (Vandegehuchte and Janssen, 2011). Epigenetic effects seem especially essential in asexual organisms because meiotic recombination is lacking. Epigenetic inheritance to some extent could take over the variation-generating function of the sexual reproduction (Verhoeven and Preite, 2014). Additionally, information on the parents' environment through epigenetic effects could be adaptive to sessile organisms or to organisms which have low mobility (Salinas et al., 2013; Verhoeven and Preite, 2014). The collembolan species *F. candida* fits both conditions. *F. candida* is a parthenogenetic, soil living arthropod (Fountain and Hopkin, 2005) and being an euedaphic Collembola species it has low mobility (Hopkin, 1997). The stability of epigenetic modifications could be different between genes (Salinas et al., 2013; Verhoeven and Preite, 2014), which could affect carry-over of the parental information. Similar to our transgenerational effect, when a blowfly (*Protophormia terraenovae*) was fed with copper-contaminated food not only the exposed animals reacted with an elevated immune response and slower growth but so did the untreated offspring generation (Pölkki et al., 2012).

The effect of etofenprox on all measured parameters was more pronounced in the multi-transgenerational than in the transgenerational exposures. This could be attributed to the additive parental and grandparental impact. Similar to the poor food availability for *F. candida* over two generations, which affected the number of reproductive events (Hafer et al., 2011), the effect of etofenprox seemed to accumulate over generations. In the parent generation the smaller egg

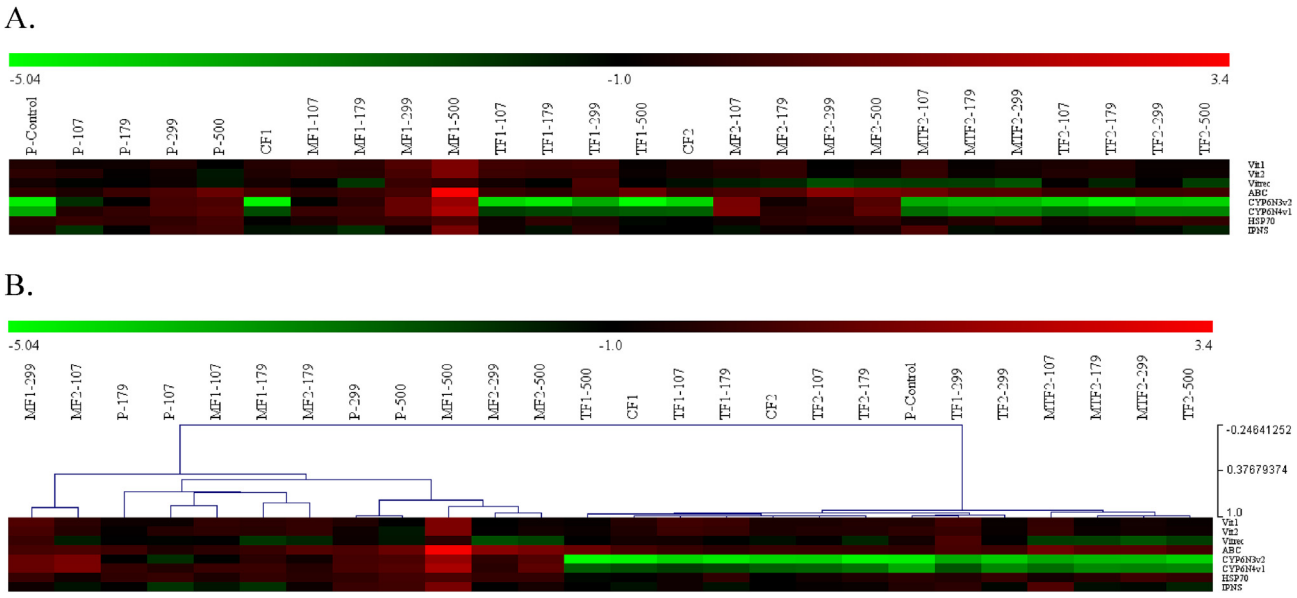

Fig. 5. A: heatplot of the logarithm of the gene expression data for *Folsomia candida* exposed to etofenprox (in the formulation Trebon® 30 EC) in LUFA 2.2 soil. B: heatplot of the logarithm of the gene expression data with clustered treatments. vit1: vitellogenin, vit2: vitellogenin-like protein, vitrec: vitellogenin-receptor, ABC: ABC-transporter, CYP6N3v2: cytochrome oxidase 6N3v2, CYP6N4v1: cytochrome oxidase 6N4v1, HSP70: heatshock protein 70, IPNS: isopenicillin-N-synthetase. The etofenprox concentrations are in mg/kg dry soil. P: parents, TF1: first generation transgeneration treatment, MF1: first generation multigeneration treatment, TF2: second generation transgeneration treatment, MF2: second generation multigeneration treatment, MTF2: second generation multi-transgeneration treatment, C: control.

number and the higher egg volume suggest that the collembolans increased their energy input into a single offspring rather than maximising the offspring number. The negative correlation between the egg size and egg number is a well-known phenomenon (Szabó and Bakonyi, 2017; Tully and Ferrière, 2008). Crommentuijn et al. (1997) found that the egg number of *F. candida* decreased with increasing concentration of the insecticide chlorpyrifos and the fungicide triphenyltin hydroxide, but they did not measure egg size. Consequently, it is not known how much energy the parent springtails invested into the offspring in that study.

Hence, it could conceivably be hypothesised that with the increased input of energy into a single egg, the total energy invested in reproduction could be decreased to the advantage of survival and detoxification. Moreover, larger eggs usually result in bigger offspring. The bigger offspring could be more vital, maximising the fitness by this strategy change (Tully and Ferrière, 2008). Furthermore, the egg-size could decrease because of the common trade-off between survival and reproduction (Fox et al., 1997; Fox and Czesak, 2000; Mousseau and Fox, 1998). While in the F1 generation the egg number and volume were reduced by etofenprox, in F2 there was a hormetic effect, which could be the result of a kind of adaptation, maybe through epigenetic imprinting. As shown by Tully and Ferrière (2008), the reproductive strategy is quite flexible in *F. candida*, enabling this species of fast and large adjustments of their reproductive traits.

In the parent generation, the collembolan population almost went extinct at the highest etofenprox concentration. The survivors, however, became increasingly less susceptible in the MF1 and MF2 generations, as seen from the higher survival and juvenile number LOEC. This could be due to epigenetic imprinting or because of a bottleneck which selected the most adapted animals. Similar to our results, *Myzus persicae* aphids, exposed to a hormetic imidacloprid concentration, were less susceptible in four subsequent generations and had higher survival than the parent generation (Rix and Cutler, 2018). de Lima e Silva et al. (2018) observed a small decrease in the toxicity of the neonicotinoid thiamethoxan to *F. candida* throughout a three-generation test. Multigeneration exposure to nano silver and silver-nitrate exposure over ten generations caused sensitisation of *Caenorhabditis elegans*, with reproduction of the population decreasing even stronger in later generations (Schultz et al., 2016). Leon Paumen et al. (2008) revealed that after exposure of *F. candida* to phenanthrene for three generations, mortality and reproduction did not change significantly. But after the fourth generation, the animals at the highest exposure concentration became very sensitive and did no longer reproduce. By contrast, our results show decreasing sensitivity, most probably because of the strong early selection in the parent generation, which could select the more resistant individuals.

The recommended field dosage of the insecticide ranges from 0.15 to 0.6 L Trebon® 30 EC/ha (approximately 0.3–1 mg/kg etofenprox in the top 1 cm of the soil). In our experiment, much higher concentrations were used and in the lowest concentrations we did not find any long-term effects. Therefore, application of this insecticide according the manufacturers suggestions most probably will not harm *F. candida* populations in the field.

4.2. Gene expression

The decreased amount of total RNA, which stretched over one non-treated generation both in the trans- and multi-transgenerational treatments, suggests generally lower health of the animals due to etofenprox exposure. Salinas et al. (2013) separated three different types of multigenerational effect, which may be a good framework to explain some of our results. The first one is when the parental environment influences the offspring's phenotype, but there is no plasticity in reacting to the current environment. In this case, the phenotype and the environment could be decoupled. The second type is when both parental effect and plasticity of the trait influence the phenotype, but there is no interaction between them. The third type is when the parental effect and phenotype plasticity are interacting.

In the parent generation, the stress genes, ABC-transporter, cytochrome-oxidases, and the heat-shock protein were up-regulated, which is in accordance with earlier observations showing an increased need for detoxification and stress response (Holmstrup et al., 2014; Nota et al., 2009). In contrast, expression of the IPNS gene responded differently. At the lowest etofenprox concentration the gene was down-regulated, but at higher concentrations it was activated in a dose-related manner, and it was overexpressed compared with the control at the two highest concentrations. There are several possible explanations for this result. First, the formulation Trebon® 30 EC has an antimicrobial effect, which makes it possible for the animals to spend energy on detoxification rather than on producing antibiotics. Some xenobiotics may make animals more susceptible to infections, e.g. phenanthrene was shown to activate antimicrobial genes (Nota et al., 2009) and diclofenac to upregulate immunity-related genes (Chen et al., 2015). Second, in cases of milder stress, the animals are activating different genes than IPNS, which explains the lower expression at the lower etofenprox concentrations and use of the β-lactam system again at higher concentrations. This scenario is possible, as in the case of the phenanthrene and diclofenac exposures of *F. candida* the IPNS gene was not upregulated (Chen et al., 2015; Nota et al., 2009). In the multigeneration exposure the expression of all stress genes, except for IPNS, showed a strong dose-dependent pattern. Both in the F1 and F2 transgenerational exposures, HSP70 showed a transgenerational increase with increasing etofenprox concentration. This could mean a stable heritable epigenetic modification (Verhoeven and Preite, 2014).

It is possible to classify the HSP70 gene expression pattern to the first epigenetic category of Salinas et al. (2013), while between different treatment groups, in the F2 generation there seemed no difference in the expression of HSP70.

CYP6N4v1 expression was dose-related increased in the parent generation. In F1 transgenerational treatment, CYP6N4v1 was not affected by etofenprox, but in F2 the transcription showed a decrease with increasing grandparental concentration. This phenomenon can be explained in different ways. First, the negative slope of TF2 CYP6N4v1 gene expression compared to the parent generation's positive slope could be a random epigenetic modification (Verhoeven and Preite, 2014). But if it is a random modification, it cannot explain the stable trend which also is visible in the F2 generation. Second, this expression pattern is part of the third epigenetic category defined by Salinas et al. (2013), where epigenetic pattern and environment do interact, so that the patterns could be different in case of juvenile exposure (F1 generation) and no exposure at all (F2 generation). This was the case in the research of Hafer et al. (2011) who found some accumulation of the effects of poor food availability, but in their study the environmental effect was stronger than the maternal or grandparental impact.

The MTF2 generation responded quite similar to the TF2 treatment: HSP70 expression increased, while CYP6N4v1 expression showed a decrease with the parental (F1) exposure concentration. Usually the CYP6N4v1 transcription level increases with increasing exposure level as was the case for phenanthrene and cadmium exposure (de Boer et al., 2013) or following exposure to a municipal landfill soil (Roelofs et al., 2012). The cause of the decreased expression could be similar as in TF2: an epigenetic modification in a random manner or a type three epigenetic modification according to Salinas et al. (2013). The expression of MTF2 showed increased stress with an increasing ABC-transporter expression. Our result agrees with earlier findings, where cadmium exposure raised the expression of ABC-transporters that transport harmful products out of the cell (Chen et al., 2014).

The reproductive genes reacted to the insecticide stress according to the well-known energy allocation scheme (Congdon et al., 2001). In the parent and MF2 generations, vitellogenin-receptor transcription decreased with increasing etofenprox concentration. While this gene jointed not only with vitellogenin transportation but also with reaching

maturity (Cong et al., 2015), it suggests that the collembolans probably reached maturity later at the higher etofenprox concentrations. The number of eggs also decreased with the increase of the egg volume. The dose-related response of vitellogenin receptor and egg volume supports the hypothesis that the parents invested more energy into individual offspring. Similar results were found for another formulation of etofenprox (Trebon® 10 F): with increasing concentration, the egg volume increased while the egg number remained constant, which means that the collembolans increased the investment into single offspring (Szabó and Bakonyi, 2017). In the F1 generation of the multigeneration treatment, the increased expression of vitellogenin and vitellogenin-like protein, without increasing egg number or egg volume, suggests that the vitellogenin and vitellogenin-like protein were utilized as an antioxidant (Corona et al., 2007; Park et al., 2018). Corona et al. (2007) found that in queen honeybees, vitellogenin, a precursor of the protein of egg yolk, was also an antioxidant which improved bee longevity. Queen bees (*Apis mellifera*) are both long-lived and fertile, whereas workers, derived from the same genome, are both relatively short-lived and normally sterile. Old queens showing much higher vitellogenin expression than workers were more resistant to oxidative stress (Corona et al., 2007).

This result suggests that vitellogenin may also be an antioxidant in *F. candida*. In the F2 generation of the multigeneration exposures, the egg volume and egg number showed a hormetic increase at the lower etofenprox concentrations. This is possibly not a consequence of vitellogenin transcription, which was not affected by etofenprox exposure in the F2 generation. Rather the intensive transcription of other stress genes could have caused the hormetic increase in reproduction. When *Myzus persicae* was exposed to low doses of imidacloprid, reproduction was stimulated. This hormetic response had its trade-offs and led to lower reproduction in the subsequent generations. The total reproduction over generations became higher at the hormetic concentrations compared to the control (Ayyanath et al., 2013).

The treatment groups MF2 and MTF2 showed a difference in fitness-maximising strategies. At a lower etofenprox concentration, the multigeneration group invested more energy into single offspring, so the juveniles had more chance to survive if they were exposed later on (Tully and Ferrière, 2008). The multi-transgeneration group rather invested energy in producing more offspring. The disappearance of the positive effect on egg number at higher concentrations could be due to the additive effect of the exposures over different generations. We hypothesize that collembolans at the higher etofenprox concentrations had to invest more energy into detoxification. The increased expression of stress genes showed that collembolans did not have enough energy to invest into offspring. Similar results were found for zinc-exposed *Daphnia magna* populations. Vandegehuchte et al. (2010b) found that the stress genes were upregulated while the juvenile number and size decreased compared to the control group in the F1 and F2 generations. Another possibility is that our results are early signs of resistance, as was found for *M. persicae* exposed to low concentrations of imidacloprid, which could withstand the treatment better after four generations (Rix and Cutler, 2018). Four year feeding of Bt-maize to *F. candida* changed some life-history parameters (larger eggs, higher growth rate) compared to the isogenic counterpart (Szabó et al., 2017).

We speculate that the dose-related increase of HSP70 and decrease of CYP6N4v1 expression in the TF2 group was an epigenetic event. Nevertheless, there is a small chance that the mentioned changes in HSP70 and CYP6N4v1 expression are the consequence of germ cell exposure of F1 animals. While the germ cells already form in the early stage of embryonic development (Hopkin, 1997), they could be affected by etofenprox in exposed F1 juveniles, and through this, the F2 generation may also be influenced. However, in our opinion, this effect could not be significant. First, survival and juvenile numbers were markedly less affected in the MTF2 and TF2 than in the MF2 treatment, which suggests that the insecticide effect on MTF2 and TF2 was not very strong. Second, if the more sensitive parameter, the gene expression, was inspected then the difference from the multigeneration treatment was also visible. The transgenerational group showed a decreased CYP6N4v1 expression and upregulation of HSP70, while in the multigenerational treatment most stress genes and vitrec were also noticeably affected. So, even if the germ cells would have been affected by etofenprox, this should not have had a significant effect on the F2 generation. However, further research is needed to investigate the transgenerational effects of etofenprox on the molecular mechanisms of *F. candida* reproduction. Epigenetic modifications can lead to insecticide resistance (Brevik et al., 2018), but the molecular mechanisms of these processes are poorly understood.

5. Conclusions

The insecticide etofenprox, in the formulation Trebon® 30 EC, showed both transgenerational and multigenerational effects on *F. candida*. In the transgenerational treatment, effects on survival and reproduction were observed in the F1 generation, but this finding may be due to direct exposure of the F1 juveniles to the insecticide. Apparent transgenerational effects of etofenprox on the transcription of HSP70 and CYP6N4v1 were found in the F2 generation. The HSP70 expression was upregulated and the CYP6N4v1 down-regulated in a dose-related manner without direct pesticide exposure. Further study of the molecular mechanisms behind the observed HSP70 and CYP6N4v1 expression changes is required. Multigenerational effects are expressed as a reduced effect on reproduction (juvenile numbers) at F1 and F2 generations. Cellular responses to etofenprox appeared both in trans- and multigenerational treatments, and showed high sensitivity. The obtained amount of RNA was decreased in all treatments in a dose-related manner (except in TF2 generation), suggesting poor health of the etofenprox-exposed collembolans. The stress-connected genes reacted with a dose-related increase to etofenprox exposure, which suggests that the population could be more resistant to further insecticide exposure or other environmental stress factors. The transcriptomic patterns of the stress genes could indicate an etofenprox stress of the population. The HSP70 gene indicates general stress, but the CYP6N4v1 gene is connected with the detoxification of ester bond-containing xenobiotics. The activated genes in the transgenerational treatment and the reduced inhibition of reproduction in the multigenerational treatment both resemble an early stage of resistance or acclimation.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.03.052.

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