SZENT ISTVÁN UNIVERSITY
PHD SCHOOL OF BIOLOGICAL SCIENCES

**PHD DISSERTATION THESIS**

# TRANS- AND MULTIGENERATION EFFECTS OF TREBON PESTICIDES ON *FOLSOMIA CANDIDA* WILLEM 1902 (COLLEMBOLA)

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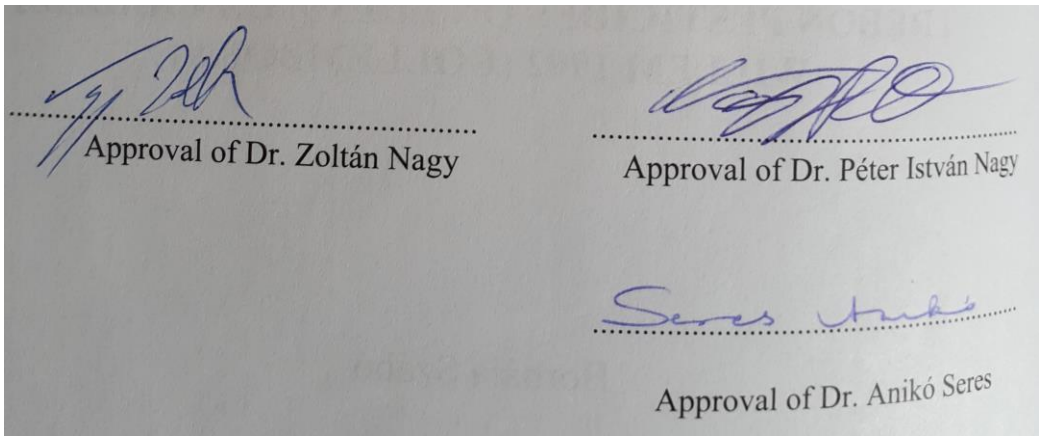

# 1. Introduction

The intensive use of pesticides caused unwanted side effects that could have not been revealed with the old supervising technics. That is why the study of pesticide effects on non-target species become necessary. Contemporary, it is an obligatory element of the authorization process in Europe. Every pesticide has to go through earthworm, water flea, fish, bird, and rat tests. These experiments are acute test most of the time while the chronic tests are only obligatory for vertebrates. Thus, standard acute tests are not suitable to reveal the long-term effects of any pesticide. The sublethal tests measure reproduction parameters and life-strategy parameters beside mortality that are better extrapolated to long-term. These parameteres could be e.g. time of maturity, heart rate, growth, egg size. The multigeneration, and transcriptomic tests are more and more widespread in the research process. The use of these tests reveals the effects appearing generations later, such as resistance, extinction, etc. There is a need for multigeneration tests in the environmental risk assessment.

Pyrethroids were extracted first from the Chrysanthemum plant pollen and were used as insecticides. The mode of action is the blockage first of all of the sodium, potassium, calcium and chlor ion-channels, causing strong hyperactivity. The continuous fireing of the neurons cause convulsions that usually causes the death of the animal, but recovery also occur. Trebon pesticides, as Trebon 10 F and Trebon 30 EC contains the active substance etofenprox, which is a pyrethroid-type insecticide. Trebon formulations are used widely, and could be used in different crops against several kinds of arthropods. The effects of etofenprox on non-target species, such as springtails, are not well known

Springtails are cosmopolitan animals, could be found anywhere at the Earth except the open-water of seas and oceans. Springtails are important in the decomposition process, significant preys of the soil predators; they spread and regulate the mycorrhiza. Springtails fill a key role in the soil foodwebs. That is why agricultural treatments, such as pesticide application could have serious consequences to the agroecosystem functioning if springtail populations are influenced.

Springtails are frequently used model animals in ecotoxicological studies. Mortality, reproduction parameters (egg size, egg number, egg shape, etc.), maturity, clutches, and behaviour are measured to reveal the effects of xenobiotics. These tests use the dose-dependency curve (EC10, EC50, LOEC, NOEC) to specify toxicity. In the sublethal tests, growth and reproductive success are usually used in the case of the springtails. The carryover effect over generations is a significant factor because if more generations' fitness is

decreased due to the sublethal effects on the parents, then the damage of the population is more severe than a one-time acute pollution.

The most popular ecotoxicology model species among springtails is *Folsomia candida* because in contrast with the other model springtails, such as *Folsomia fimetaria* or *Orchesella cincta,* it reproduces asexually and easy to rear in the laboratory. That is the reason why it is very suitable for multigeneration experiments.

Epigenetical variance is especially important for asexual and low mobility species while the natural variance generating process cannot work because of the parthenogenesis or the high inbreeding. However, the epigenetic mechanisms could partially take over the genetic variance generating function. *F. candida* is a parthenogenetic and low mobility species, that is why it could be important subject if epigenetic patterns should be taken into account in ecotoxicological studies.

**1.1 Aims of the dissertation are:**

- to quantify the effects of Trebon 10 F, and Trebon 30 EC on the mortality, and juvenile number of the *F. candida*
- to test the effect of Trebon 30 EC insecticide on the egg number, and egg size of the *F. candida* with the stress and reproductive gene expression through three generations.
- to test the effect of Trebon 30 EC on the behaviour of *F. candida* in an acute test.
- to test the trans- and multigeneration effects of Trebon 10 F, and Trebon 30 EC on the life-history parameters and behaviour of *F. candida.*

# 2. Material and methods

## 2.1 Measurement of egg parameters

The egg volume and shape were measured and calculated in the same way in every experiment. The clutches on the plaster of Paris were spread with a fine brush then digital photos were made under a stereomicroscope. The shortest and longest diameter relative at a 90° angle to each other were measured with the aid of the ImageJ software.

The volume of each egg was calculated according to the prolate spheroid's volume formula $V = 4/3\ \pi \times a \times b^2$, where "a" is the longer and "b" is the shorter diameter. The cube root of the volume was used in statistical models to reach normal distribution. The longer diameter divided with the shorter diameter was used as the ratio of egg diameters, so the ratio was always a number between 0 and 1.

## 2.2 Trebon 10 F insecticide reproduction and food-choice experiment

Based on the results of the dose-dependency experiment and the concentration used at orchards and forests, 0.882 ml Trebon 10 F/L water concentration was chosen for further work. The latter mean concentration, the tenth thinner and tenth concentrated solutions were used in a parent-offspring reproduction and food-choice test. There was a control group kept on Petri dishes, and another in OECD soil.

Factorial experiment design was used. In every treatment group, three parameter were measured: food-choice, egg counting, egg volume. A food-choice test, counting of the eggs and measure of egg parameters were also performed.

Synchronized, 10-12 days old individuals (n = 90 per treatment) were placed in plastic boxes. The boxes were filled with 24.5 g of dry OECD soil and mixed with 5.5 ml of pesticide solution or tap water. Altogether, four boxes were used, three for the treatments plus one for the control. After 20 days, all of the 90 individuals were carefully separated from the soil, and 30 animals were chosen randomly from each treatment. These 30 collembolans were placed individually in Petri dishes with plaster of Paris. This transport induced egg laying in most cases. The boxes were opened for aeration, feeding (ad libitum), and cleaning the mould if needed once each week. Each animal was kept alone during the measurements and was assigned an identification number. On the ninth day after transporting, the clutches were spread carefully with a wet brush and a digital photo was taken of each (Olympus C7070 Wide Zoom camera with Olympus C5060 ADL optic). The eggs were numbered on the photo. Thereafter, ten eggs were chosen randomly from each clutch for measurement.

From the adult parents, 25 were chosen to the food-choice test from every treatment group. The food-choice test was performed in mini Petri-dishes with 4 cm diameter and 1 cm height. One layer wet filter paper was laid to keep up humidity, and one more paper to show the places of the food and feaces counting. Grinded maize leaf (Zamora) and baker's yeast (standard food) were offered as food. Both food types were put on the target marks of the filter paper at the start of the experiment, and the animal was placed in the middle of the Petri-dish to have equal chance to get to each food type. In one Petri-dish, one animal was left for a week, then feaces in the target marks were counted.

For the reproduction experiment, the hatched offspring of the individually kept parents in all 30 Petri dishes were separated into two equal groups. The first group was handled the same way as the parents. This treatment was regarded as the multigenerational effect of the insecticide (M0,1, M1; M10). The second group of collembolans was exposed to no further insecticide treatments. This treatment was regarded as the transgenerational effect (T0,1, T1, T10; only the parental effects manifest). Collembolans were left for 20 days in the Petri dishes without repeated insecticide treatment. Thereafter, thirty offspring were placed individually in new Petri dishes then the eggs were measured as described above. Also, 25 five adults were chosen from every treatment group for the food-choice test.

### 2.3 Trebon 30 EC insecticide trans- and multigeneration transcription experiment

Based on the results of the dose-dependency test's EC50 value, the following concentrations were chosen: 107, 179, 299 and 500 mg active substance /kg dry LUFA soil and a control receiving demineralized water. The lowest concentration used in this experiment is near to the manufacturer's suggestions for apple, pear, and quince orchards. The soil was spiked half a day before use. Thirty-gram moist soil was placed in each jar, with ten replicates in the control and five for each test concentration.

The OECD 232 *Folsomia candida* reproduction test was used as the basic design for the ecotoxicological part of the experiment. Every generation was handled with identical manner. The animals were 10-12 days old at the start of every 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 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 experiment, 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. 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 part of the experiment, 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. 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.

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 minutes for more optimal DNA degradation. RNA concentration was measured on a NanoDrop ND-1000 spectrophotometer (Wilmington, DE, USA) and stored at -80°C. 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 and succinate dehydrogenase.

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. 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. 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.

### 2.4 Trebon 30 EC insecticide trans- and multigeneration life-history experiment

The experiment was performed in 9 cm diameter Petri dishes filled with a 0.5 cm layer of plaster of Paris and powdered graphite (20µm Sigma-Aldrich ®) (8:1) mixture. While graphite is an inert material it will not bind Trebon 30 EC after treatment. The following concentrations were used to wet the Petri-dishes: 0, 0.766, 1.303, 2.215, 3.765 and 6.4 ml Trebon 30 EC/L water.

In the parent generation, 15 collembolan/concentration was used. The animals were kept individually. The laid egg clutches were transferred to a clean Petri dish, where the clutches were spread with a fine wet brush. The spread clutches were photographed to count and measure the size of them. The following egg parameters were measured: clutch number, size of the first clutch, time of maturation (egg laying time of the first clutch), total egg number, egg volume, the ratio of egg diameters, total reproduction investment (total number of eggs multiplied by the mean egg volume). Ten days after laying eggs repeated photo were taken from the spread clutches to check unhatching ratio (number of unhatched eggs/ number of laid eggs). Therefore, if unhatching ratio grows than the number of unhatched eggs is higher, so the individual is less successful. Estimation of the food consumption measured as the animals were fed ad libitum on a target-patterned filter paper. The grazing was measured by counting the number of faeces around the food in a ring-shaped sector of 2 cm. The filter paper was switched after two weeks. Switching of filter paper was necessary to prevent moulding which would have impeded faeces counting. Faeces were photographed and counted. The number of faeces was the measure of food consumption.

Twice a week photos were taken of the animals too. The body length of the animals was measured from the front of the head to the end of the last

abdomen segment. Two measurements were taken from photos immediately after each other. The mean of the two measurements was regarded as the body length of the animal. The experiment was 21 days long so the animals were 32-34 days old at the end of the experiment. The animal's length in the beginning and the end of the experiment was measured, and the absolute growth (body length at the end minus at the start of the experiment) was calculated.

On day 21 a behaviour test was carried out on the adults according to the acute behaviour test. The individuals were transferred into a clean Petri-dish, in the centre of a plastic ring (15 mm diameter). Video record was performed. The movement of each collembola within the arena was recorded for two minutes. The total times of locomotion activity, average velocity during locomotion activity, and the energy invested in movement were determined. The trade-off between the growth and reproduction were tested with a trade-off rate (absolute growth divided by total reproduction investment). We assumed that if the trade-off rate increases than more energy are allocated to the growth, but if it decreases than more energy is allocated to reproduction.

When the juveniles of the parent generation become 10-12 days old, they were divided into two groups. One group was put into clean Petri-dishes individually (transgeneration group, T) and the other were put into treated Petri-dishes individually (multigeneration group, M). From these juveniles were separated the member of the F1 generation. The animals were handled as the parent generation and the measurements were carried out in the very same way. 12-12 collembolan were kept individually in every concentration and treatment group. In F2 and F3 generation, the offspring of the multigeneration group were put into insecticide-treated Petri-dishes individually; the offspring of the transgeneration group were kept on insecticide-free Petri-dishes.

# 3. Results

## 3.1 Trebon 10 F insecticide reproduction and food-choice experiment

In the P generation, concentration did not affect the egg number. The increase of the Trebon 10 F concentration significantly decreased the egg volume, and the ratio of egg diameters. The egg number was not significantly affected either in F1 transgeneration or in multigeneration group. The egg volume significantly increased with the Trebon 10 F concentration. In the transgeneration group, this is only an increasing trend; the concentration groups did not differ significantly from the control.

The 95% percent confidence intervals gained from the evaluation f the food choice test were shifted into positive directions, so the mean differences between the faeces number around the two types of food shifts to the yeast preference. If the groups treated with the same concentration are compared, then it is visible that the preference of the control is very variable. The preference values of concentration lower than the field concentration (P0,1, T0,1, M0,1, TT0,1) are around 0, so these groups did not prefer a food type. The intervals of the field concentrations (P1, T1, M1, TT1) are rather at positive values, so these groups prefer yeast. The interval of the first treated generation (M1) is clearly at the positive values. Among the tenth concentrated groups (P10, T10, M10, TT10), P10 and T10 preferred yeast. In the case of M10 and TT10 groups were no preference detected. Compared to the parents the intervals of the F1 generation offsprings shifted to the right (positive values). In the F2 generation, none of the treated groups had a preference.

## 3.2 Trebon 30 EC insecticide trans- and multigeneration transcription experiment

Significant dose-response of the mortality was found in the case of P, F1M, F2M and F2MT treatments. Mortality was most affected in the treatment of F2M, where the insecticide concentration of 179 mg/kg caused a significant decrease. The juvenile number of P treatment was lower than in the other ones due to the shorter duration of this part of the experiment. Significant dose-response was found in the P, F1M, F1T and F2M treatments. The most pronounced effect was found in the P treatment, where as low as 107 mg/kg insecticide concentration caused a significant (79%) decrease in the juvenile number.

In the P generation, the expression of ABC and both CYP6N3v2 and CYP6N4v1 genes increased with increasing pesticide concentration (LOECs 500 and 107 mg/kg, respectively). The expression of HSP70 gene showed a slight but non-significant increasing trend with increasing exposure concentration. In the MF1 generation, the expression of both CYP6N3v2 and CYP6N4v1 genes was increased with the Trebon 30 EC concentration (LOEC

107 mg/kg) and HSP70 (LOEC 179 mg/kg). In the TF1 generation, only the expression of HSP70 gene increased with increasing etofenprox concentration (LOEC 179 mg/kg). In the MF2 generation, the expressions of ABC, CYP6N3v2, CYP6N4v1, and HSP79 genes increased with increasing pesticide concentration; LOECs were 299, 107 and 179 mg/kg, respectively. In the MTF2 generation, ABC gene expression was significantly higher at 107 mg/kg than in the control (approx. 250%). CYP6N3v2 gene expression slightly but not significantly increased with increasing exposure concentration. CYP6N4v1 expression decreased while HSP70 gene expression increased with increasing pesticide concentration; LOECs were 179 mg/kg in both cases. In the TF2 generation, the expression of HSP70 increased while CYP6N4v1 gene expression decreased with the increasing pesticide concentration; LOEC was 299 and 500 mg/kg, respectively.

The expression results clearly reveal that stress-related genes showed increased responses along the multigenerational treatment. The transgenerational treatment caused a constitutive activation of HSP70 and a decrease of the CYP6N4v1 gene expression level. The multi-transgenerational treatment responded similar to the TF2 treatment, except that MTF2 also showed activated CYP6N3v2 gene expression. During the multigenerational treatment, ABC, CYP6N3v2, CYP6N4v1, and HSP70 genes were markedly activated.

### 3.3 Trebon 30 EC insecticide trans- and multigeneration life-history experiment

In the parent generation, in the case of the absolute growth, hormesis was detected in a piece-wise linear model. Mortality significantly increased dose-dependently, but there was no difference from the control.

In the F1 multigeneration group, there was no significant dose-dependency in the starter length. The animals of the highest concentration group were significantly longer than that of the control group. The total number of eggs was significantly decreased by the treatment. The groups of the two highest concentration laid significantly fewer eggs than the control. There was a 50% elevated hormesis in the total reproduction investment in the lowest concentration. Mortality significantly increased dose-dependently, but there was no difference from the control. The growth-reproduction trade-off dose-dependently increased, but there is no significant difference from the control. In F1 transgeneration group, the starter length has shown a significant dose-dependency. Similarly to the multigeneration group, the animals descended from the highest concentration group were longer at the start of the experiment. The absolute growth was significantly lesser in the highest concentration group compared to the control.

In the F2 multigeneration group, the starter length and the final length was significantly dose-dependent. The highest concentration group started the experiment with a smaller length, and they finished the generation with the smaller length too. There was no difference in absolute growth. The total number of eggs was significantly decreasing with concentration. In the 1.3 ml/L concentration group, 23% hormesis was found, while the animals in the highest concentration group laid significantly fewer eggs than the control. The number of clutches also significantly decreased with the concentration. In the 1.3 ml/L concentration group, 20% hormesis was found, while the animals in the highest concentration group laid significantly fewer clutches than the control. The piece-wise linear model estimated the time of maturation earlier in the first four concentration than the control, but the highest concentration maturated later. The total reproduction investment was decreasing with the concentration; the investment of the highest concentration group was decreased. Mortality increased with the concentration, and in the highest concentration, the mortality was significantly higher than in the control. The highest concentration group went extinct. The food consumption was increasing with the concentration. The trade-off ratio was increasing with the concentration. In the highest concentration group, much more was invested in growth than in the control. In the F2 transgeneration group, only unhatching ratio and food consumption have shown significant dose-dependency. The unhatching ratio significantly decreased with the concentration, but there was no significant difference from the control. The three highest concentration groups have consumed more than the control.

In the F3 multigeneration group, the egg volume decreased with the increase of the concentration, but there was no significant difference compared to the control. The unhatching ratio was increasing with the concentration, and significantly less egg hatched in the two highest concentration group. In the F3 transgeneration group, the starter length dose-dependently decreased. The final length was decreasing dose-dependently; the highest concentration group have grown less. The absolute growth decreased with the concentration; the highest concentration group had a smaller absolute growth. The total number of eggs and the number of clutches dose-dependently increased. In the 2.2 ml/L and 6.4 ml/L concentration groups, both parameters were greater than the control. The time of maturation was earlier in every concentration group. The egg volume has shown mild hormesis (16%) in the case of the first two concentration. The total reproduction investment was dose-dependent and was greater in the case of the highest concentration than the the control. The trade-off rate was decreasing with the concentration. This increased investment is significant in the case of the highest concentration.

# 4. Discussion

## 4.1 Trebon 10 F insecticide reproduction and food-choice experiment

Inverse relationship between the total number of eggs and egg size in insect species is a common phenomenon because of the trade-off between production and reproduction. However, a dose-response relationship was not found between Trebon 10 F and the number of *F. candida* eggs, neither in the parent nor in the offspring populations. The most probable reason for this is that the tested concentrations were out of the effective range.

Modification of the egg size due to Trebon 10 F application showed different results than that of the egg production. In the case of the parent generation, the insecticide decreased the egg size in a concentration-dependent manner. This is in agreement with earlier findings which showed that low reproductive investment is presumed if the environmental conditions are poor. The negative influence of Trebon 10 F on embryonic development was shown in this study by the decrease in the ratios of the egg diameters. This indicates that the eggs were less spherical as the insecticide concentration increased in the parent generation. The egg shape of F. *candida* is related to the viability, as spherical eggs were less viable when compared to spheroid eggs. This finding can be a sign of the energy trade-off between the costs of defence mechanisms for the parent individuals against the Trebon 10 F application versus investment into offspring.

Completely different results for the effects of the insecticide on the egg size and shape were found for both the T and M offspring generations. Neither the number of eggs nor the ratio of the egg diameters changed, but the egg size increased as the Trebon 10 F concentration increased. This is a clear sign that the insecticide exposure disturbed the epigenetic status of the parent generation in a concentration-dependent manner. Results such as these have rarely been found in ecotoxicology. In addition, egg size positively correlated with the viability of the *F. candida* offspring. Epigenetic variation induced by pesticides can be manifested. Nevertheless, the true mechanisms of the information transmission in the case of *F. candida* due to Trebon 10F application are still to be revealed. If only the parent generation of collembolan were exposed to the insecticide (group T) the outcome was very similar, as in the multigenerational treatment. This is straightforward evidence that in this study the transgenerational effect of the insecticide Trebon 10 F exists. Etofenprox, the active substance in Trebon 10 F has a high octanol/water partition coefficient (log Kow of 6.9), which suggests the possibility of bioaccumulation in fat (e.g. *F. candida* egg cytoplasm and developing embryo). If this is the case, the significant effect of Trebon 10 F on egg size can be explained. However, the mechanisms of etofenprox action during collembolan embryonic development are not yet identified.

The data presented in this study show that Trebon 10 F has multigenerational and transgenerational effects on the egg traits of *F. candida*, and it seems that the size of the egg is the trait, which is primarily affected. Moreover, these effects are dose-dependent.

Based on the results of the parent generation, my hypothesis is that the control group consume more maize because, after monodietic yeast feeding, the animals try to take up some nutrients what is in higher amount in the maize leaf. However, in the first offspring generation, there was no preference in the control group, what debates this hypothesis. The dose-dependency in the two generations indicates that the insecticide-treated animals need more better quality food (baker's yeast in this case), than the control group. Thus, the fitness of *F. candida* is the highest when fed with the preferred food. Based on our unpublished data, the baker's yeast is much better quality food for *F. candida* than the maize leaf.

In the second offspring generation, I wished to reveal whether two generation is enough to make the effects of the insecticide fade. One generation was not enough to make the effect cease, because some transgeneration effects remained.

### 4.2 Trebon 30 EC insecticide trans- and multigeneration transcription experiment

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. Apparently, the animals were able 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 resistance, or the resilience of the population against Trebon 30 EC.

The transgenerational effect on the survival and reproduction of *F. candida*, observed in the F1 generation, may be explained by epigenetic imprinting or the transmission of the pesticide through egg content. The effect of etofenprox on all measured parameters was more pronounced in the multigenerational than in the transgenerational line. This could be attributed to the additive parental and grandparental impact, which seemed to accumulate over generations. In the parent generation, the smaller egg number and the higher egg volume suggest that the collembolans increased their energy input into a single offspring to maximise the offspring number. 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, maximizing the fitness by this strategy change. Furthermore, the egg-size could decrease

because of the trade-off between survival and reproduction. While in the F1 generation the egg number and volume were reduced by Trebon 30 EC, in F2 generation there was a hormetic effect, which could be the result of epigenetic imprinting. 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 Trebon 30 EC 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 effect, which selected the most adapted animals.

The scientific literature separates three different types of epigenetic effect. The first one is when the parental environment influences the offspring's phenotype, but there is no plasticity 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 influences the phenotype, but there is no interaction between them. The third type is when the parental effect and phenotype plasticity are interacting. In ecotoxicology, this type of categorization have got no attention yet, but it is a good framework to explain some of our results. The stability of the epigenetic effect could be different between genes, what could influence the information submission to the offsprings.

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. In contrast, expression of the IPNS gene responded differently. At the lowest Trebon 30 EC 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 and diclofenac to upregulate immunity-related genes. Second, in cases of milder stress, the animals are activating different genes than IPNS, which explains the lower expression at the lower Trebon 30 EC concentrations and use of IPNS again at higher concentrations. 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 Trebon 30 EC concentration. This could mean a stable heritable epigenetic modification.

It is possible to classify the HSP70 gene expression pattern to the first epigenetic category, 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 generation the transcription showed a decrease with increasing grandparental concentration. This phenomenon can be explained in different ways. First, the negative slope of TF2 generation CYP6N4v1 gene expression compared to the parent generation's positive slope could be a random epigenetic modification. However, 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, 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).

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. 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. The expression of MTF2 gene 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.

The reproductive genes reacted to the insecticide stress according to the well-known energy allocation scheme. 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, it suggests that the collembolans probably reached maturity later at the higher Trebin 30 EC concentrations. 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 in the Trebon 10 F reproduction and food-choice experiment: with increasing concentration, the egg volume increased while the egg number remained constant, which means that the collembolans increased the investment into single offspring. 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.

In the F2 generation of the multigeneration exposures, the egg volume and egg number showed a hormetic increase at the lower insecticide concentrations. This is possibly not a consequence of vitellogenin transcription, which was not affected by the exposure in the F2 generation. Rather the intensive transcription of other stress genes could have caused the hormetic increase in reproduction.

The treatment groups MF2 and MTF2 showed a difference in fitness-maximising strategies. At a lower Trebon 30 EC concentration, the multigeneration group invested more energy into single offspring, so the juveniles had more chance to survive if they were exposed later on. The multi-transgeneration group rather invested energy in producing more offspring. Thus, some offsprings will survive in case of pollution too and the population could reach the original size fast. 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 insecticide concentrations had to invest more energy into detoxification. The increased expression of stress genes showed that collembolans did not have enough energy to invest in offspring. Another possibility is that our results are early signs of resistance.

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, they could be affected by Trebon 30 EC in exposed F1 juveniles, and through this, the F2 generation may also be influenced. However, in my 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 groups 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. Therefore, even if the germ cells would have been affected by Trebon 30 EC, this should not have had a significant effect on the F2 generation. Epigenetic modifications can lead to insecticide resistance, but the molecular mechanisms of these processes are poorly understood.

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 genes 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 (based on juvenile number LOEC) at F1 and F2 generations. 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. Thus, the populations are able to gain back the previous population size if there is only one pesticide treatment.

### 4.3 Trebon 30 EC insecticide trans- and multigeneration life-history experiment

Multigeneration and transgeneration changes have been found in the life-history of *F. candida* caused by Trebon 30 EC. The morality was weak in the parent generation, but the total reproduction investment decreased and hormesis have been found in the absolute growth. The decrease of investment into reproduction to decrease mortality is a general reaction in case of invertebrates. The investment into growth could be a defensive reaction, while the bigger offspring/individual has a greater fitness. After the mild effects in the parent generation, F1 transgeneration group have shown a mildly decreased growth and in F2 generation the food consumption was increased. The results suggest that the effects of the over carrying of decreased fitness fades, and the effects of the insecticide getting into the eggs also fades away.

In the multigeneration group, the following effects became more severe compared to the parents: the number of eggs decreased, so did the total reproduction investment, mortality increased and the animals rather invested into the growth than the reproduction. These results are in agreement with previous findings that the investment into reproduction decreases when the environmental conditions are adverse. This was the trend in the F2 generation too when the slight reproduction and the great mortality caused the highest concentration group to extinct. This phenomenon proves the accumulation of the effects; the gradual decreasing of fitness drove the group to extinction. The stress-decreased reproduction of *F. candida* is a known phenomenon. The Trebon 10 F experiments or the gene expression study conducted with Trebon 30 EC also have shown a similar pattern. The F3 multigeneration line have shown a very different picture compared to the previous generation. After the extinction of the highest concentration group, in the multigeneration group the egg volume decreased a little, but there was no other pesticide effect. The

groups treated with lower concentrations have become more resistant. This result is similar to our previous experiment with Trebon 30 EC.

In the F3 transgeneration group, the compensation of the treatment effects on the parent generation has begun. The animals were generally smaller (the starter length, the final length, and the absolute growth were smaller) and the total reproduction investment was increased, compared to the control. The trade-off rate was favouring the reproduction and the animals were more active. This reproduction-growth trade-off is fitting the general energy allocation scheme. If *Daphnia magna* exposed to zinc, the stress genes are upregulated, and the reproduction is decreased in the next two generations because of the trade-off between detoxification and reproduction. In the current experiment, the opposite could be in the background when after two untreated generations the stress gene activation could be decreased based on our gene expression experiment with Trebon 30 EC, so there is enough energy for reproduction again.

The life-history pattern of the F3 generation suggests the possibility of epigenetic inheritance. The resistance of the multigeneration line could be the result of the selection of the epigenotypes or the changing of the epigenetic patterns. The results of this experiment are similar to the gene expression experiment.The animals get close to extinction in the higher concentration, but in the multigeneration line, the animals have become more resistant (visible in the juvenile number). In that experiment, the effect on the transgenerational line began to fade, but the CYP6N4v2 gene in the F2 generation upregulated again, which seems to support the epigenetic pattern hypothesis.

### 4.4 Conclusions

Generally, both Trebon 10 F and Trebon 30 EC insecticides are harmless on the *F. candida* springtail populations in the manufacturer recommended concentrations. More severe side-effects, such as extinction or the transgenerational activation of the stress genes, were observed only in much higher concentrations than the recommended. Both before mentioned severe side effects were observed in the case of Trebon 30 EC, so probably Trebon 10 F is less harmful to *F. candida*. In the life-history parameter experiment, there was egg volume decrease even in F3 generation in the multigeneration line, and a strong compensating process started in the transgeneration line. These phenomenons suggest the stronger effect of the Trebon 30 EC comparing to the Trebon 10 F.

Both pesticides cause a decrease of the reproduction in the parent generation, what could be the consequence of the investment into the detoxification, and general stress response as the transcription measurements showed. In the case of Trebon 10 F, the increased investment into the reproduction was observed in both trans- and multigeneration lines. In the

Trebon 30 EC experiment, the multigeneration group decreased investment into reproduction to increase growth in a dose-response manner. The comparison of Trebon 10 F and Trebon 30 EC also suggests that Trebon 30 EC has a stronger effect on *F. candida* than Trebon 10 F because of more energy invested into survival than reproduction in the first case. The different formulation most probably causes the different effects of the two insecticides. The multigeneration experiments suggest in case of both pesticides epigenetic effects, which could be measured in the reproductive parameters and the transcriptomes.

In the life-history experiment, the fourth generation has brought results with critical importance. These data suggest that four-generation long experiments let us assume the long-term effects on *F. candida*, so it would worth to study the consistency of this pattern. If the four-generation effect is indeed consistent, then the standardization of such a test and introduction into the risk assessment would clearly a great blank spot.

# 5. New scientific results

- The Trebon 10 F and Trebon 30 EC do not cause permanent damage in *Folsomia candida* populations when the recommendations of the manufacturer are respected. However, Trebon 30 C causes a decrease in the reproduction in the parent generation, but the population is able to recover in the long-term.

- The effect of Trebon 10 F is disappearing until the F2 generation; there is no further effect on food-choice.

- The Trebon 30 EC is more toxic to *F. candida* than Trebon 10 F. Because Trebon 30 EC causes the individuals to invest rather into the growth than to the reproduction. The transcription experiment proved that the stress and detoxificating genes are intensively upregulated.

- Based on the dose-dependent induction of the reproduction and growth parameters in the transgenerational group it is presumable that both Trebon 10 F and Trebon 30 EC have epigenetic effects on *F. candida*.

- Trebon 30 EC activates heatshock protein and cythochrome-oxidase 6N4v2 genes dose-dependently even after two generations raised in clean soil. This could be the result of the selection of the epigenotypes or a stable epigenetic modification.

# 6. Scientific publications

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