THESIS OF THE PHD DISSERTATION

Neonicotinoid-induced behavioral and biochemical changes in two-humped flea crab (*Dikerogammarus villosus*) test animals

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I. BACKGROUND AND OBJECTIVES

Currently, environmental problems are very complex. One of these challenges is the food crisis caused by overpopulation. The use of various plant protection agents (pesticides) has now become part of our everyday life. These compounds play important roles in agriculture, both for increasing yield and for reducing the costs of food production. They help intensive farming and support defense against various pathogens. Moreover, chemical residues accumulated in the soil and underground water bases as a result of excessive pesticide use can enter surface water bodies through meteorological and geological processes, where they can accumulate through adsorption mechanisms or desorb under suitable conditions. If pesticides are present in natural waters in large quantities and in a form that can be absorbed by living organisms, the natural balance of local ecosystems and the food chain can be upset. In extreme cases, this can also lead to a narrowing of habitats and a decrease in biodiversity. Therefore, it is particularly important to study the concentration-dependent physiological effects of neonicotinoids, the most widely used insecticides in the world, on individual invertebrate and vertebrate species. The negative effects that occur are most evident in terrestrial invertebrate species. The long-standing destruction of bee colonies around the world is clear evidence of this phenomenon. On land, plant protection agents can enter organisms more easily and in orders of magnitude greater quantities than they can in aquatic environments. In addition, invertebrates are significantly more sensitive to contamination with this group of pesticides. Therefore, the examination of invertebrate primary-secondary consumer organisms located at lower levels of food chains can provide insight into the effects of a foreign substance on a given ecosystem. However, little is known about aquatic invertebrate species in this regard. We do not have sufficient quantity and quality of information to be able to take effective environmental protection measures and create

legislation. With respect to neonicotinoids, insufficient fish tests (e.g., firefish [*Pimephales promelas* Kendall, 1903], bluegill sunfish [*Lepomis macrochirus* Rafinesque, 1819], spotted catfish [*Ictalurus punctatus* Rafinesque, 1818], rainbow trout [Oncorhynchus mykiss (Walbaum, 1792)], or large water flea (Daphnia magna Straus, 1920)) have been conducted because these species are not sensitive enough to accurately study the behavioral and biochemical effects of the mentioned group of compounds, i.e., they only respond to a relatively high concentration range. In this dissertation, which is highly sensitive to neonicotinoid contamination, the two-humped flea crab (*Dikerogammarus villosus* (Sowinsky, 1894)) from Balaton serves as a test organism, through which we gain insight into the processes, the knowledge of which and the results of the tests carried out on the experimental subject can provide missing data and knowledge for the examination of both the ecotoxicological profile of substances and their environmental risk assessment. Owing to their widespread use and persistence, neonicotinoids are often detected as soil pollutants; however, recently, their detection frequency has been high, and their concentration has varied within wide limits (ng/l-µg/l). Therefore, long-term ecological effects are not expected. Although the clarification of the role of neonicotinoids in the mass destruction of pollinators and newly developing bee diseases has become the center of intensive research, we have less data for aquatic nontarget organisms. This prompted us to investigate invasive species that occur in large numbers in Lake Balaton, the largest freshwater shallow lake in Central Europe.

Questions to which we sought answers:

1. What behavioral changes do the most common neonicotinoid active substances cause in the test animal D. villosus?
2. What biochemical changes lead to changes in social and individual behaviors?

Set goals:

1. To examine the long-term toxic effects on aquatic test organisms under laboratory conditions at low concentrations. Our measurement endpoints are the conventionally often used lethality and/or immobility, reproductive capacity, and sublethal effects (e.g., movement, feeding activity).

2. Key biomarkers that play important roles in the detoxification process, including oxidative stress and changes in MXR activity, were observed.

3. The amounts of active components and degradation products were determined via modern analytical-chemical methods.

II. EXPERIMENTAL METHODS USED

The experimental samples were collected from the littoral region of Lake Balaton on the Tihany Peninsula. We kept the flea beetles in unfiltered aerated Balaton water in 10-liter tubs containing medium-sized stones and quagga shells. They were fed carrots ad libitum. The behavioral studies were based on recorded short films and analysed with Fiji ImageJ software. For the MXR measurements, the total protein content of the individuals was determined via the Bradford method. GST activity was determined according to the Sigma-Aldrich method. AChE activity was determined via the Ellman method. Statistical analyses were performed using OriginPro software.

III. THESIS-LIKE SUMMARY OF RESULTS

In our tests, we examined different active ingredients of neonicotinoid insecticides. These were KLO, IMI and TIAK. In the case of KLO and TIAK, commercially available molded products were also exposed until 2018 and 2020, respectively. We did not obtain access to an agent containing IMI. The investigated endpoints were immobility duration and swimming activity in terms of behavior and GST and AchE enzyme activity tests in biochemical terms. APACS 50 WG significantly reduced the immobility duration of the experimental subject at a concentration of 3.9 ng/l. Behavioral testing revealed that chemically pure KLO significantly reduced immobility duration and swimming activity in addition to causing abnormal wheezing patterns in all treated groups at ambient and higher concentrations (16, 32, 48 μg/l). Compared with the control, pure IMI resulted in abnormal behaviors at concentrations higher than the ambient concentration, significantly reducing first swimming activity (20, 80 μg/l) and then immobility duration (250 μg/l). Pure TIAK significantly increased the immobility time at a concentration higher than the ambient concentration (100 μg/l), although it did not cause as much abnormality in behavior as did KLO or IMI. The Calypso 480 SC was exposed as a molded TIAK. It can also significantly increase the immobility period at a concentration

one-third lower than that of the pure agent (30-100 μg/l). In terms of biochemical aspects, the APACS 50 WG KLO-containing insecticide can cause significant chemostimulation in the MXR mechanism at a concentration of only 4.5 ng/l, even after 1.5 hours of exposure. With respect to GST, when pure KLO was exposed, no significant differences were detected among any of the treated groups (16, 32, and 48 μg/l); however, a significant increase in enzyme activity was detected at higher concentrations of pure IMI (160 and 250 μg/l). Pure TIAK did not induce significant changes in this phase of detoxification, but Calypso 480 SC at concentrations of 10 and 30 μg/l significantly increased GST activity. By determining the change in AchE enzyme activity, we showed that KLO significantly reduced AchE activity in all the treated groups (160 and 250 μg/l), whereas IMI significantly stimulated AchE activity at relatively high treatment concentrations (80, 160, and 250 μg/l). TIAK did not cause significant changes in conduction, but Calypso 480 SC had a significant blocking effect in the 10, 30 and 100 μg/l treatment groups. We have also shown that if the experimental subject cannot be fed his natural food while in captivity, then feeding with alternative foods is indeed possible, taking toxicological aspects into account and under control. In our case, the antioxidant content of the carrot food did not significantly influence the toxicological outcome of the tests.

1. APACS 50WG KLO-containing insecticide:

- at a concentration of 3.9 ng/l, during a treatment time of 3 hours, it significantly reduced the duration of immobility of the experimental subjects
- at a concentration of 4.5 ng/l during a treatment period of 1.5 hours, it significantly enhanced the functioning of the MXR cellular defense mechanism

2. Pure KLO:

- significantly reduced immobility time and swimming activity in all treatment groups (16, 32, 48 µg/L)
- did not result in significant changes in GST enzyme activity in any of the treated groups (16, 32, 48 µg/L)
- significant inhibition of AChE enzyme activity in all treated groups (16, 32, 48 µg/L)

3. The pure IMI:

- in lower treatment concentrations (20, 80 µg/L) the swimming activity was significantly reduced, while the immobility time during exposure in more concentrated solutions
- in more concentrated media (160, 250 µg/L) it caused a significant increase in GST enzyme activity
- significantly stimulated AChE activity in more concentrated solutions (80, 160, 250 µg/L)

4. The TIAK-containing Calypso 480 SC insecticide:

- induced changes similar to the pure form, however even a medium with a concentration of 30 µg/l resulted in a significant increase in the immobility duration
- significantly stimulated GST activity in treatments with concentrations of 10 and 30 µg/l
- significantly inhibited nerve conduction in the groups treated with concentrations of 10, 30, 100 µg/l

5. The pure TIAK:

- compared to the control, up to a treatment concentration of 100 µg/l, it increased the immobility duration in the treated individuals to an increasing extent, and at 100 µg/l

- did not significantly affect detoxification by GST in any of the treated groups (10, 30, 100, 250 µg/L)

- no significant change in AChE activity occurred in any of the treated groups (10, 30, 100, 250 µg/L)

IV. THE IMPORTANCE OF RESEARCH

In the course of this doctoral (PhD) research, we studied adult individuals of *Dikerogammarus villous* crayfish, a superior species of crayfish found in large numbers in Lake Balaton (Central Europe's largest shallow water lake), in terms of the degree of behavioral and biochemical changes caused by short-term exposure to various neonicotinoid insecticides. We chose this species because it is not considered a target organism for the tested substances, and until now, it has been the subject of few ecotoxicological tests worldwide. Our results show that D. villosus is very sensitive to contamination by neonicotinoid compounds, similar to the nontarget species reported in the literature. To the best of our knowledge, aquatic crayfish and insect organisms are the most sensitive to neonicotinoid exposure. This means that long-term (months, years) exposure can lead to large-scale damage and the disappearance of ecosystems. Therefore, D. villosus would be suitable for use as a model organism. To the best of our knowledge, this is the first series of studies in which ecotoxicological testing of neonicotinoid insecticides was performed on the experimental subjects we used during acute treatments at environmentally relevant and relatively high concentrations. With our work, we would like to draw attention to the importance of ecotoxicological tests with aquatic test animals, as well as to the importance of increasing the selectivity of plant protection agents and the search for sensitive species. The regular revision of environmental risk assessments and surface water guidelines can serve as a tool for these goals.

V. SCIENTIFIC PUBLICATIONS RELATED TO THE TOPIC OF THE DISSERTATION

- Somogyvári, D., Vehovszky, Á., Farkas, A., Horváth, R., and Győri, J. (2020). Multimarker approach for the evaluation of environmental impacts of APACS 50WG on aquatic ecosystems. Invertebrate Neuroscience 2020 20:4, 20(4), 1–3. https://doi.org/10.1007/S10158-020-00254-2. IF: 1,38

- Somogyvári, D., Farkas, A., Mörtl, M., and Győri, J. (2022). Behavioral and biochemical alterations induced by acute clothianidin and imidacloprid exposure in the killer shrimp, Dikerogammarus villosus. Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology, 261: 109421. https://doi.org/10.1016/j.cbpc.2022.109421. IF: 4,39

VI. SCIENTIFIC PUBLICATIONS RELATED TO THE TOPIC OF THE DISSERTATION

- 1st Symposium on Invertebrate Neuroscience, 2019. augusztus 13-17, Tihany
  Multimarker approach for the evaluation of environmental impacts of xenobiotics on aquatic ecosystems. David Somogyvári, Ágnes Vehovszky, Anna Farkas, János Győri MTA Ökológiai Kutatóközpont, Balatoni Limnológiai Intézet

- IX. Ökotoxikológiai Konferencia, 2019. November 22, Budapest
  Multiparaméteres megközelítés a rovarölő szerek környezeti hatásának értékeléséhez a *Dikerogammarus villosus* felemáslábú rákfaj példáján. Somogyvári Dávid, Farkas Anna, Vehovszky Ágnes, Győri János

- Tudományos Konferencia, 2020 September 16, Veszprém
  Különböző környezetszennyezők (rovarölő szerek, nanoanyagok, endokrin diszruptorok) környezeti hatásának vizsgálata vízi ökoszisztémákon. Somogyvári Dávid

- Pannon Tudományos Napok - Magyar Tudomány Ünnepe, 2020. November 16-17.
  A neonikotinoidok környezeti hatásának vizsgálata vízi ökoszisztémákon. Somogyvári Dávid

- X. Ökotoxikológiai Konferencia, 2020. December 4.
  Az Apacs 50 WG hatásának vizsgálata a *Dikerogammarus villosus* rákfajon. Somogyvári Dávid

- XI. Ökotoxikológiai Konferencia, 2021. November 26.
  A clothianidin és az imidacloprid hatásának vizsgálata kétpúpos bolharák *(Dikerogammarus villosus)* célszervezeten. Somogyvári Dávid, Farkas Anna, Győri János

- TOX'2022 Tudományos Konferencia, 2022.10.12-14, Zalakaros
  Neonikotinoid-indukált viselkedési és biokémiai változások a *Dikerogammarus villosus* tesztállatban. Somogyvári Dávid

VII. OTHER ANNOUNCEMENTS NOT DIRECTLY RELATED TO THE TOPIC OF THE DISSERTATION

- Ács, A., Komáromy, A., Kovács, A. W., Fodor, I., Somogyvári, D., Győri, J., A., Farkas. (2023). Temperature related toxicity features of acute acetamiprid and thiacloprid exposure in implications on reproductive performance. Comparative Biochemistry and Physiology, Part C, 268: 109601 https://doi.org/10.1016/j.cbpc.2023.109601

- Farkas, A., Somogyvári, D., Kovács, A. W., Mörtl, M., Székács, A., and Győri, J. (2022). Physiological and metabolic alterations induced by commercial neonicotinoid formulations in *Daphnia magna*. *Ecotoxicology*, *31*(3), 415–424. https://doi.org/10.1007/s10646-022-02520-y