Research Article

Zebrafish Embryo Neonicotinoid Developmental Neurotoxicity in the FET Test and Behavioral Assays

Rebecca von Hellfeld[1,2], Viktoriia Ovcharova[1,3], Samantha Bevan[4], Maria-Agapi Lazaridi[4], Caroline Bauch[4], Paul Walker[4], Susanne Hougaard Bennekou[5], Anna Forsby[6] and Thomas Braunbeck[1]

[1]Aquatic Ecology and Toxicology, Centre for Organismal Studies, University of Heidelberg, Heidelberg, Germany; [2]Current address: School of Biological Sciences, University of Aberdeen, Aberdeen, United Kingdom; [3]iES Landau, Institute for Environmental Sciences, University of Koblenz-Landau, Landau, Germany; [4]Cyprotex Discovery Limited, Cheshire, United Kingdom; [5]National Food Institute, Technical University of Denmark (DTU), Lyngby, Denmark; [6]Department of Biochemistry and Biophysics, Svante Arrhenius Laboratories for Natural Sciences, Stockholm University, Stockholm, Sweden

Abstract

The need for reliable, sensitive (developmental) neurotoxicity testing of chemicals has steadily increased. Given the limited capacities for routine testing according to accepted regulatory guidelines, there is potential risk to human health and the environment. Most toxicity studies are based on mammalian test systems, which have been questioned for low sensitivity, limited relevance for humans, and animal welfare considerations. This increased the need for alternative models, one of which is the zebrafish (*Danio rerio*) embryo. This study assessed selected neonicotinoids at sub-lethal concentrations for their effects on embryonic development and behavior. The fish embryo acute toxicity test (OECD TG 236) determined the lowest observable effective concentrations, which were used as the highest test concentrations in subsequent behavioral assays. In the FET test, no severe compound-induced sublethal effects were seen at < 100 µM. In the coiling assay, exposure to ≥ 1.25 µM nicotine (positive control) affected both the burst duration and burst count per minute, whereas ≥ 50 µM thiacloprid affected the mean burst duration. Exposure to ≥ 50 µM acetamiprid and imidacloprid induced significant alterations in both mean burst duration and burst count per minute. In the swimming assay, 100 µM acetamiprid induced alterations in the frequency and extent of movements, whilst nicotine exposure only induced non-significant changes. All behavioral changes could be correlated to findings in mammalian studies. Given the quest for alternative test methods of (developmental) neurotoxicity, zebrafish embryo behavior testing could be integrated into a future tiered testing scheme.

1 Introduction

Therapeutic disasters such as the thalidomide tragedy (Franks et al., 2004; Vargesson, 2015, 2019) are the most prominent examples of a long list of drug scandals[1]. Together with "environmental accidents" like Minamata disease caused by mercury poisoning (Health & Chemicals, 2002; Kitamura et al., 2020), these fatal errors underlined the need for stricter regulations and more in-depth testing to prevent effects of drugs on the developing brain and nervous system. Neurotoxicity (NT) has since been defined as biological, chemical, or physical agent-induced adverse effect(s) on the structure or functioning of the nervous system (Cunha-Oliveira et al., 2008). Developmental neurotoxicity (DNT) has frequently been defined as an inhibition or alteration of the nervous system development leading to irreversible changes (Delp et al., 2018). Whilst DNT due to chemical exposure during fetal development is commonly observed in early life, one cannot exclude neurological symptoms after fetal exposure only presenting in adult individuals (Dubovický et al., 2008). Thus, the timepoint of exposure and the timepoint of

Received November 2, 2021; Accepted February 21, 2022;
Epub February 23, 2022; © The Authors, 2022.

ALTEX 39(3), 367-387. doi:10.14573/altex.2111021

Correspondence: Thomas Braunbeck, PhD
Im Neuenheimer Feld 504
69120 Heidelberg, Germany
(braunbeck@uni-hd.de)

Rebecca von Hellfeld, PhD
23 St Machar Drive
Aberdeen AB24 3UU, Scotland, UK
(rebecca@vonhellfeld.de)

[1] The list of drug scandals started in 1937 with the sulphanilamide catastrophe (Paine, 2017), and from then, included a long list of drugs such as Acomplia, Avandia, Buflomedil, Clinafloxacin, Clobutinol, Coxigon, Enoxacin, Fleroxacin, Gatifloxacin, Gemifloxacin, Grepafloxacin, Lipobay, Lomefloxacin, Lumiracoxib, Mibefradil, Nefazodon, Pefloxacin, Sparfloxacin, Temafloxacin, Trasylol, Trovafloxacin, Valdecoxib, Vioxx, Ximelagatran, and Zelmac.

DNT endpoint observation are not necessarily the same. Examples of DNT endpoints are changes in the migration or interaction of cells and structural deformations of the brain and/or nervous system (Chang, 1998; Aschner et al., 2017).

Usually, assays to determine the (D)NT potential of a compound are based on mammalian model organisms, e.g., the DNT study in accordance with the Organization for Economic Co-Operation and Development (OECD) Test Guidelines (TG) 426 (OECD, 2007) and 443 (OECD, 2018). Whereas these *in vivo* protocols based on one- or two-generation animal experiments are very time- and cost-intensive, modern *in vitro* models such as the NeuriTox or the PeriTox tests assess well-defined aspects of the nervous system (Hoelting et al., 2016; Delp et al., 2018). Along with increasing concerns regarding the sensitivity of mammalian test systems for the determination of potential risks to human health (Bailey et al., 2014; Aschner et al., 2017; Fritsche, 2017; Monticello et al., 2017; Clark and Steger-Hartmann, 2018), and specifically DNT effects (Paparella et al., 2020), the quest for alternative test methods following the 3Rs principles to reduce, refine, and replace animal testing (Russell and Burch, 1959) has increased the demand for new approaches to (D)NT testing.

As one potential candidate for alternative test systems, the zebrafish (*Danio rerio*) embryo is receiving increasing attention in the scientific and regulatory community, since it combines the benefits of an *in vivo* model with compatibility with European animal welfare legislation (EU, 2010; Strähle et al., 2012) and the 3Rs principle (Russell and Burch, 1959). In practical terms, the zebrafish embryo is more time-, cost- and resource-efficient than classic mammalian models and can be made accessible for high-throughput methods (Braunbeck et al., 2015).

Neonicotinoids ("new-nicotine-like pesticides"; EFSA, 2013), a group of insecticides originally advertised for high target-specificity, are a group of chemicals associated with DNT in rodents. Second to glyphosate, neonicotinoids have continued to be the best-selling pesticides globally (Sparks and Nauen, 2015), although recently their use in Europe has been severely restricted (Auteri et al., 2017). In insects, neonicotinoids bind to the nicotine acetylcholine receptor (nAChR), resulting in altered nerve excitation, paralysis, and ultimately death (Tomizawa and Casida, 2005). Evidence suggests that neonicotinoids also adversely affect pollinating insects, birds, as well as mammals, finally disrupting entire ecosystems (Tomizawa and Casida, 2005; Millot et al., 2017; Pisa et al., 2021). Studies into the effect of neonicotinoids in aquatic vertebrates have documented impaired neurotransmission (Zhang and Zhao, 2017) along with early-life and long-term effects on neurodevelopment and behavior (Crosby et al., 2015).

For the detection and quantification of early effects of neuroactive compounds, behavioral assays have frequently been employed, since they provide insight into changes associated with nervous system functioning through movement and behavior (Klüver et al., 2015). Technical progress has allowed for sophisticated recording coupled with subsequent computer algorithm-based data analysis. Zebrafish behavioral patterns have been studied in great detail (Kalueff et al., 2013) in both embryos and adults (Bass and Gerlai, 2008; Rihel et al., 2011; Zindler et al., 2019a,b, 2020), providing a solid basis for the assessment of behavioral changes elicited by chemical exposure. The earliest locomotor behavior in the zebrafish embryo is spontaneous tail coiling, which is initiated 17 hours post-fertilization (hpf) and based on spontaneous neuronal firing before becoming a controlled cyclical movement to support hatching by 72 hpf (Kimmel et al., 1974). Following a strict developmental timeline (see Fig. S1[2]), the coiling behavior has been found to be affected by various compounds in a mode of action (MoA)-specific manner (Kokel et al., 2010; Raftery and Volz, 2015; Vliet et al., 2017; Zindler et al., 2019b). Usually, a distinction is made between post-hatching early swimming behavior and later mature beat-and-glide swimming patterns (Saint-Amant and Drapeau, 1998; Drapeau et al., 2002). Due to the susceptibility to toxicants, the so-called basal swimming activity assay has frequently been used to study behavioral endpoints at later developmental stages (Letcher et al., 2010; Basnet et al., 2019).

The purpose of the present study was to analyze the potential of the coiling assay and the swimming assay to detect (D)NT-related effects of neonicotinoid exposure. Exposure during critical early life-stages was hypothesized to trigger behavioral changes in zebrafish embryos at concentrations too low to cause overt morphological teratogenic effects. Due to structural similarities of neonicotinoids with nicotine, similar behavior alternations such as concentration- and time-dependent hyper- and hypoactivity or anxiety responses were expected (Freeman et al., 1987; Bencan and Levin, 2008). Therefore, effects of acetamiprid, clothianidin, dinotefuran, imidacloprid, thiacloprid and thiamethoxam were compared to those of nicotine, a known developmental neurotoxicant (positive control). The upper limits of test concentrations in the coiling assay (Zindler et al., 2019b) and the swimming assay (Zindler et al., 2020) were determined as the concentrations causing 10% sub-lethal effect (EC_{10}) in the OECD fish embryo acute toxicity (FET) test (OECD, 2013).

2 Animals, materials and methods

2.1 Zebrafish handling and maintenance

Adult "Westaquarium" strain zebrafish were obtained from the breeding facilities at the Aquatic Ecology and Toxicology Group (Centre for Organismal Studies, University of Heidelberg, Heidelberg, Germany; license no. 35-9185.64/BH). Fish maintenance,

Abbreviations
ACh, acetylcholine; ANOVA, analysis of variance; DCA, 3,4-dichloroaneline; DMSO, dimethyl sulfoxide; (D)NT, (developmental) neurotoxicity; EC, effective concentration; FET, fish embryo acute toxicity test (OECD TG 236); hpf, hours post fertilization; LC, lethal concentration; MS, mass spectrometry; MW, molecular weight; nAChR, nicotinic acetylcholine receptor; OECD, Organization for Economic Co-operation and Development; SC, step change; TG, Test Guideline.

[2] doi:10.14573/altex.2111021s1

Tab. 1: Concentration range in µM tested in the FET test, the coiling assay (21-47 hpf), and the swimming assay (83-120 hpf)

Compound	FET test	Coiling assay	Swimming assay
Acetamiprid	6.25-100	2.50-100	2.50-100
Clothianidin	6.25-100	–	–
Dinotefuran	6.25-100	–	–
Imidacloprid	6.25-100	2.50-100	–
Nicotine	6.25-100	1.25-25	1.25-25
Thiacloprid	6.25-100	2.50-100	–
Thiamethoxam	6.25-100	–	–

–, not tested in the respective assay: Since clothianidin, dinotefuran and thiamethoxam did not induce increased spontaneous movements at 24 hpf in the FET test, they were not tested further in the coiling assay. Acetamiprid was selected for the swimming assay as a proof of concept.

breeding conditions and egg production was conducted based on internationally accepted standards (Lammer et al., 2009). For all assays, OECD TG 236 standardized water was prepared freshly in-house. For the behavioral assays, the embryos were reared in crystallizing dishes. In brief, freshly laid eggs (< 1 hpf) were transferred to 50 mL crystallizing dishes filled with the respective test solutions. Total medium renewal was done daily (semi-static exposure) and, where recordings were part of the protocol, renewal was conducted between recordings, replacing the well plate at least 20 minutes prior to the next recording for re-acclimatization.

2.2 Chemicals

The neonicotinoids were distributed by the Joint Research Centre (JRC, Ispra, Italy); shipping and storage were conducted in accordance with the manufacturers' instructions. Acetamiprid, clothianidin, dinotefuran, imidacloprid, nicotine (positive control), thiamethoxam and thiacloprid were obtained from Merck (Darmstadt, Germany), whilst 3,4-dichloroaniline (DCA; used as FET test positive control) was obtained from Sigma Aldrich (Deisenhofen, Germany), and dimethyl sulfoxide (DMSO; used as solvent control) was obtained from Honeywell International (Offenbach, Germany). In the FET tests, artificial water was used as negative control, along with DMSO as solvent control. In the coiling and swimming assays, only DMSO was used as solvent control. Additional information regarding the neonicotinoids and nicotine can be found in Table S1[2]. Neonicotinoid stock solutions were prepared once at 100 mM in DMSO, aliquoted for each run and stored at -20°C until required. All medium samples were prepared with a final DMSO concentration of 0.1%; final test concentrations in the FET, the coiling assay, and the swimming assay are summarized in Table 1. During the experimental procedures, the stock solution aliquots were individually thawed and stored at 4°C.

2.3 Chemical analysis of medium samples

Mass spectrometry (MS) sample preparation

For each replicate and concentration (including negative and solvent controls), 2 mL of the exposure medium was collected at the end of the 120 hpf exposure and immediately frozen in liquid nitrogen along with 2 mL freshly prepared stock. The samples were stored at -80°C until shipping on dry ice for chemical analysis.

Compound quantification by liquid chromatography-mass spectrometry (LC-MS)/MS

Samples containing the tested neonicotinoids were analyzed on a system consisting of an Acquity™ Binary Solvent Manager (BSM), an Acquity™ 4 position heated column manager, 2777 Ultra High Pressure Autosampler, and a Xevo TQ MS Triple Quadrupole mass spectrometer (Waters Ltd, Herts, UK). The analysis was performed using an Acquity™ HSS T3 column (1.8 µm) 2.1 x 30 mm (Waters Ltd, Herts, UK) fitted with a SecurityGuard™ ULTRA Fully Porous Polar C18 cartridge (Phenomenex, Cheshire, UK). The column was maintained at 40°C, and the injection volume was 4 µL; details are given in Table S2[2].

2.4 Experimental procedures

FET test

The acute toxicity of the test substances was determined according to OECD TG 236 (OECD, 2013) with 3 biological replicates utilizing eggs from different breeding groups, which where thus biologically distinct from one another. Each biological replicate was run with 10 eggs per treatment group, which here are termed as "technical replicates". These definitions also apply to the coiling and swimming assays. Collected eggs were individually transferred into 24-well plates (TPP, Trasadingen, Switzerland) containing 2 mL of fresh test solution at room temperature per well by 2 hpf latest. The well plates had been pre-exposed to the respective concentration medium to account for compound adsorption. Subsequently, well plates were sealed with self-adhesive foil (SealPlate® by EXCEL Scientific; Dunn, Asbach, Germany) to avoid evaporation and placed into an incubator at 26 ± 1°C set to a light/dark cycle of 14/10 h. All developmental alterations of the embryos were documented at 24, 48, 72, 96, and 120 hpf after the daily total medium renewal. FET tests with a minimum mortality rate of 30% in the positive control

(4 mg/L DCA) and a maximum sub-lethal effective rate of 10% (i.e., EC_{10}, in accordance with the OECD TG 236: OECD, 2013) in the negative control (standardized water; Lammer et al., 2009) at 96 hpf were classified as valid. Endpoints were recorded as defined by von Hellfeld et al. (2020). Examples of typical alterations induced by exposure to nicotine are given in Figure S2[2].

Coiling assay (21-47 hpf)
Embryos were initially treated as outlined for the FET test (3.3.1). Five 7 h old zebrafish embryos per well were transferred into a 24-well plate (TPP) containing 2 mL fresh test solution (3 biological replicates with 20 technical replicates each). The well plates had previously been pre-exposed to the respective concentration medium to account for compound adsorption. The embryos were centered with a 5.3 mm diameter polytetrafluoroethylene ring (ESSKA, Hamburg, Germany). The localization of the test concentrations within the 24-well plate was randomized; each plate contained four test concentrations as well as a solvent control (0.1% DMSO; negative control). The plate was placed on an acrylic glass-covered light box (twelve infrared lights, 880 nm, 40°, 5 mm, Knightbright, Taiwan) within a HettCube 600R incubator (Hettich, Tuttlingen, Germany) at 26 ± 1°C set to a light/dark cycle of 14/10 h, equipped with a large water reservoir to prevent medium evaporation from the open well plate. The first dark phase covers the beginning of the coiling assay (until 23.5 hpf), followed by the light phase (between 23.5 and 37.5 hpf). The second dark phase covers the end of the coiling assay time (from 37.5 hpf onwards). Videos with a duration of 8 min (mpeg-4, 25 frames/s) were taped between 21 and 47 hpf (camera: Basler acA1920-155µm, Ahrensburg, Germany; lens: M7528-MP F2.8 f75mm, Computar®, Basler, Ahrensburg, Germany; Filter: RG850 heliopan, Gräfelfing, Germany) utilizing the Ethovision® Software (Noldus, Wageningen, Netherlands). To avoid unnecessary movement of the recording set-up through the vibrations of the capacitor, the incubator was switched off for 15 minutes every hour (3 min prior to the onset of recording) as outlined by Zindler et al. (2019a). Lighting was not affected by the hourly incubator switch-off. An example video is provided as Video S1[3]. Software settings for the coiling assay can be found in Table S3[2].

Swimming assay (83-120 hpf)
Acetamiprid was selected for the swimming assay as a proof of concept. Embryos were initially treated as outlined for the FET test (3.3.1). At an age of 72 h, one embryo per well was transferred into 96-well plates (TPP) containing 360 µL fresh test solution per well (3 biological replicates with 19 technical replicates each for nicotine; 2 biological replicates with 19 technical replicates each for acetamiprid). The well plates had previously been pre-exposed to the respective concentration medium to account for compound adsorption. The well plate was then placed in the DanioVision® Observation Chamber (Noldus, Wageningen, Netherlands) and thermostatically controlled throughflow (DanioVision® external Temperature Control Unit) ensured a constant water temperature of 26 ± 1°C and a light/dark cycle of 14/10 h. Videos with a 15-minute duration were recorded every hour between 83 and 120 hpf using EthoVision Software (Noldus, Wageningen, Netherlands) (Zindler et al., 2019a). The videos were then analyzed with DanioScope® Software (Version 1.1). An example video is provided as Video S23. Software settings for the swimming assay can be found in Table S3[2].

Mammalian data
The PubChem database was consulted for data with the test compounds from mammalian studies, and all studies listed for mammalian models were assessed. Where key words such as "developmental alterations/abnormalities", "embryo toxicity", "behavior", "gait", "anxiety", "activity", "motility" or similar terms were found, the findings were analyzed in-depth for exposure conditions and study duration. Additional studies were retrieved from PubMed using search keywords such as "((compound name) AND (development)) AND (mammal)", "((compound name) AND (behavior)) AND (mammal)" and "((compound name) AND (embryo)) AND (mammal)". Finally, targeted searches for specific effects observed in the FET test (such as pericardial area enlargement) were conducted. A summary of the experimental set-ups and significant findings of all referenced studies is provided in Table S4[2].

2.5 Statistical analysis

Each biological replicate was evaluated individually and compared to its corresponding control. ToxRat® (version 2.10.03; ToxRat™ Solutions, Alsdorf, Germany), GraphPad Prism version 7.03 for Windows (GraphPad, La Jolla, California, USA), and the open-source statistical software RStudio (Version 1.3.959) interface running R software (Version 4.0.2 for Windows; R Developmental Core Team, 2020) were used for statistical computing. SigmaPlot Version 14.0 (Systat Software, Erkrath, Germany) was used for data visualization. Additionally, the free software Inkscape (Software 0.92.4, 5da689c313, 2019-01-14) was used for post-editing of vector graphs. Differences from corresponding controls were considered statistically significant at $p < 0.05$.

FET test
EC_{10} and EC_{50} were calculated based on probit analysis using linear maximum likelihood regression with ToxRat® (Braunbeck et al., 2015). Here, all observed sub-lethal developmental alterations were considered for the computation of the EC values. Due to the overall low frequency of sub-lethal effects observed, however, all three replicates were assessed simultaneously with ToxRat®, thus not allowing the determination of a standard deviation (Tab. 3).

Coiling assay (behavioral changes in early-stage zebrafish embryos)
The videos were analyzed with the DanioScope Software (Version 1.1; Noldus, Wageningen, The Netherlands), and the readout data for mean burst duration [seconds] and burst count per

[3] doi:10.14573/altex.2111021s2

minute were obtained as excel spread sheets. ANOVA-on-ranks (Kruskal Wallis test) was utilized to analyze mean differences of behavior parameters versus negative controls, followed by the Dunn's *post-hoc* test against controls; data were visualized separately for each replicate, using GraphPad Prism. Time points at which at least two out of three replicates were found statistically significant ($p \leq 0.05$) were considered as an effect caused by the exposure. All significant *p*-values are listed in Table S5[2]. Furthermore, the step change (SC) in both behavioral parameters between 37 and 38 hpf was analyzed as the difference between quantified estimates at both time points to analyze the effect of light change on the zebrafish embryo behavior. The standard deviations were derived in the same way. Then the values deviating from the control group by 10% (SC_{10}) and 50% (SC_{50}), both negative and positive, were computed. The assessment of the step change is only used as a representation of the data and to determine potential trends, not as a test for a statistically significant difference between the treatment groups.

Swimming assay (behavioral changes in late-stage zebrafish embryos)

Swimming distance in the swimming assay was evaluated by summarizing the data into bins of 3 to 5 hours. Outlier screening was conducted with the R function "identify_outliers" (rstatix package, Version 0.5.0) for each biological replicate. The distance moved parameter was handled as follows: (1) The raw data was analyzed, estimates scored > 1.0% (the cut-off threshold) in "missed samples" were removed, and the remaining data were pooled into 3-5 h bins to account for the missing/removed data as well as to avoid the over-interpretation of potential differences of behavior-based biological variability rather than exposure-induced differences. An outlier check with R was implemented, followed by non-parametric analysis and Dunn's *post-hoc* test (for significant *p*-values see Tab. S6[2]). (2) The total distance moved over the recorded 25 h was estimated by summing the distance moved per individual over all time points. These data were assessed by parametric one-way analysis-of-variance (ANOVA) and multiple comparisons of means from treatment groups against a control group (Dunnett's test, package "multcomp", Version 1.4.13). Effect size was estimated in terms of eta-squared (η^2) and can be interpreted as follows: 0-0.01: no effect; 0.01-0.06: small effect; 0.06-0.14: intermediate effect; and 0.14-0.20: strong effect. For statistically significant findings, *p*-values are summarized in Table S7[2]. To determine swimming frequency, raw data were assessed in time-steps of 0.4 s and considered as a movement in events where activity was registered after a period of at least 5 seconds without movement, allowing to distinguish between actual movement and background noise. For statistically significant findings, *p*-values are given in Table S6[2].

Analysis of LC-MS/MS data

Separate calibration curves were created for each neonicotinoid by spiking water samples with known concentrations of these compounds. For all compounds, the calibration curve showed a detection threshold at low spike-in concentrations. The peak areas were adjusted by dividing the values by a machine internal standard value that was determined separately for each data point, and by dividing by the estimated volume of all organisms, resulting in a value proportional to the concentration within individuals. The limit of detection (LOD) values are summarized in Table S2[2].

Additionally, the percentage difference in actual and nominal concentrations in the fresh and 24 h old samples was computed as follows:

$$\% \text{ difference} = \frac{\text{actual-nominal}}{\text{nominal}} \times 100 \qquad \text{Eq. 1}$$

3 Results

3.1 Chemical analysis of test chemicals

The chemical analysis of actual test concentrations was conducted with freshly prepared samples as well as samples taken at the end of the 120 h experiment, when organogenesis is mostly concluded (Kimmel et al., 1995) and an increase in metabolic activity and compound interaction may be expected. Actual concentrations of acetamiprid in the 120 h samples were about 70% of the nominal concentrations in the 50 and 100 µM samples, but < 35% in the lower exposure groups (Tab. 2). The clothianidin concentration was reduced by at least 97% in comparison to the nominal concentration in all exposure groups. Dinotefuran behaved similarly to acetamiprid, with the actual concentrations in the three highest exposure groups being between 70 and 80% of the nominal concentrations and up to 37% in the remaining treatment groups. Imidacloprid showed an inverted trend compared to other neonicotinoids tested. At the lowest nominal concentration, the actual concentration was about 40% of the nominal concentration but decreased to 15% in the highest exposure group. Nicotine concentrations were found to be 51% of the nominal 100 µM, and the concentration declined to 25% of the nominal concentration in the 6.25 µM treatment group. Interestingly, the concentrations of thiacloprid and thiamethoxam after 120 h exposure were higher than the nominal concentrations. In the 50 and 100 µM medium, the actual thiacloprid concentrations were 238 and 198%, respectively. These values declined to between 20 and 32% for the lower test concentrations. Thiamethoxam was measured at 180% in the two highest exposure groups and values between 20 and 40% in the remaining three exposure groups (Tab. 2).

3.2 FET tests

Throughout any treatment, no acute mortality was observed, and EC_{50} data were determined at higher than the highest test concentration (100 µM) for all neonicotinoids tested except for acetamiprid after 120 h exposure (Tab. 3). Nicotine was the only tested compound for which the EC values could be adequately determined by ToxRat®, as more than 50% of exposed individuals showed sub-lethal effects (EC_{50}).

Following nicotine exposure, increased spontaneous movement in embryos exposed to 6.25 and 12.5 µM as well as reduced movement at the higher concentrations was observed as early

Tab. 2: Actual concentrations (µM; mean ± SD) measured in freshly prepared medium and samples collected at the end of the FET test (120 hpf) after the embryos had been exposed to the medium for 24 h (n = 3)
The percentage change between mean nominal and actual concentration of the fresh and 24 h old samples is given to the nearest integer.

	Nominal concentration (µM)									
	6.25		12.5		25		50		100	
	Fresh	24 h	Fresh	24 h	Fresh	24 h	Fresh	24 h	Fresh	24 h
Acetamiprid[a]	1.95 ± 0.13	2.20 ± 0.20	2.47 ± 0.49	3.34 ± 0.64	1.05 ± 2.12	7.52 ± 0.13	26.03 ± 0.26	35.21 ± 1.64	74.56 ± 2.79	74.67 ± 10.66
Clothianidin	-0.02 ± 0.05	0.05 ± 0.08	0.07 ± 0.08	0.21 ± 0.02	0.50 ± 0.03	0.56 ± 0.13	0.64 ± 0.15	1.57 ± 0.16	1.77 ± 0.31	2.75 ± 0.16
Dinotefuran[a]	1.23 ± 0.57	3.30 ± 0.65	2.71 ± 0.24	4.65 ± 0.13	13.48 ± 1.86	20.17 ± 0.89	34.72 ± 0.30	36.64 ± 4.82	71.12 ± 2.37	70.98 ± 11.82
Imidacloprid	1.26 ± 0.04	2.53 ± 0.19	3.05 ± 0.41	4.03 ± 0.25	3.64 ± 0.59	7.41 ± 0.29	7.45 ± 0.96	10.95 ± 0.39	13.53 ± 1.77	15.80 ± 0.38
Nicotine	2.38 ± 0.50	1.57 ± 0.22	4.41 ± 1.39	3.64 ± 0.55	15.93 ± 3.22	8.67 ± 1.93	43.84 ± 9.07	22.62 ± 3.35	73.65 ± 16.18	51.49 ± 4.38
Thiacloprid	1.62 ± 0.5	1.97 ± 0.20	2.64 ± 0.54	3.88 ± 0.29	3.52 ± 0.76	5.40 ± 0.46	42.94 ± 13.33	118.84 ± 8.26	156.80 ± 26.45	197.90 ± 22.02
Thiamethoxam[a]	1.56 ± 0.00	2.51 ± 0.90	2.69 ± 0.09	3.67 ± 0.31	3.92 ± 1.15	5.34 ± 1.05	31.99 ± 6.10	89.86 ± 11.88	98.60 ± 12.90	179.25 ± 16.54
Percentage change between the mean actual concentrations measured in fresh and 24 h old samples (%)										
Acetamiprid	-69	-65	-80	-73	-96	-70	-50	-49	-25	-25
Clothianidin	-100	-99	-99	-98	-98	-98	-99	-97	-98	-97
Dinotefuran	-80	-47	-78	-63	-46	-19	-31	-27	-29	-29
Imidacloprid	-80	-60	-76	-68	-85	-70	-85	-78	-86	-84
Nicotine	-62	-60	-65	-72	-36	-65	-12	-55	-26	-49
Thiacloprid	-74	-60	-79	-69	-86	-78	-14	+138	+57	+98
Thiamethoxam	-75	-60	-78	-71	-84	-79	-36	+80	-1	+79

[a] Fresh sample data based on 2 replicates only

as after 24 h of exposure (Tab. 4). At 48 hpf, the most frequent effect was reduced blood circulation in conjunction with blood congestion in yolk blood vessels; at 72 hpf, hatching of embryos exposed to 100 µM nicotine was delayed. In the present study, lordosis and kyphosis (Fig. S1[2]), as defined in von Hellfeld et al. (2020), were observed post-hatching in more than 50% of embryos exposed to 50 µM nicotine and in all embryos exposed to 100 µM nicotine. Further sublethal effects observed after exposure to nicotine included tremor, craniofacial deformation, and pericardial edema. Moreover, in controls, gentle tapping of the 24-well plates caused the embryos to swim towards the well walls and remain there. This response was attenuated with increasing nicotine exposure concentration, and the response was no longer observable at 100 µM, which resulted in erratic tremor and no clear directional movement.

For all neonicotinoids tested, no lethal effects as defined by OECD TG 236 (OECD, 2013) (i.e., lack of somite formation or tail detachment, coagulation, and lack of heart beat) could be recorded at concentrations up to 100 µM. Likewise, none of the neonicotinoids significantly impaired hatching. Overall, effects were observed in less than 50% of individual embryos per treatment group, leading to non-computability of certain effective concentration values in Table 3.

Despite a lack of lethal effects after exposure to the neonicotinoids for up to 120 h, various sub-lethal effects were observed (Tab. 4; Tab. S8[2]). Exposure to acetamiprid induced a slight increase in macroscopically discernible spontaneous movement at 24 hpf, followed by reduced blood circulation and congestion along with pericardial edema from 48 hpf onwards, as well as increased activity at 120 hpf. Exposure to clothianidin induced no observable effects at 24 hpf in the FET test. However, at 48 and 72 hpf a reduction in pigmentation was noted, as well a mild reduction of blood flow at 72 hpf and reduced hatching success. At 96 hpf, only mild circulatory alterations and spinal deformation

Tab. 3: EC_{10} and EC_{50} of nicotine and selected neonicotinoids (μM; based on nominal concentrations) computed with ToxRat®

	96 hpf		120 hpf	
Compound	**EC_{10}**	**EC_{50}**	**EC_{10}**	**EC_{50}**
Acetamiprid	13.7	195 $	0.3	63.8
Clothianidin	N/A	N/A	LDR	LDR
Dinotefuran	N/A	N/A	LDR	LDR
Imidacloprid	N/A	N/A	N/A	N/A
Nicotine	22.0	44.7	6.1	23.5
Thiacloprid	LDR	LDR	3.3 $	351 $
Thiamethoxam	59.2	254 $	12.4	256

LDR, effects only observed in one replicate and thus not suitable for determination of an effective concentration. $, data computed based on two replicates (no observed effects at given time point in the remaining replicate). N/A, data could not be computed by ToxRat® since no or too few effects were recorded at the given time point.

Tab. 4: List of all endpoints observed in the FET test for the neonicotinoids tested
A detailed listing of all observations and concentrations can be found in Table S8[2].

Endpoint	**Developmental timepoint**				
	24 hpf	**48 hpf**	**72 hpf**	**96 hpf**	**120 hpf**
Spontaneous movement (↓, ↓↓)	A, D, I, N, TC, TM				
Spontaneous movement (↑)	N				
Delayed hatching			A, C, D, I, N, TC, TM	N, TM	
Heartbeat (↓, ↓↓, ↓↓↓)		A, I, TM		A	N
Blood flow (↓, ↓↓, ↓↓↓)		A, I, TM		C	N
Spinal deformation (K, L)			A, C, D, I, N, TM	A, C, N, TM	A, N
Reduced body length			I, N	A, N	A, N
Edema		I	N	A, N	N
Otolith deformation					N
Pigmentation (↓, ↓↓, ↓↓↓)		A, C, D, I, N, TC, TM	A, C, D, I, N, TC, TM	A, N	
Enlarged pericardial area		A	A	A	
Craniofacial deformation			N	A, N	A, N
Reduced yolk resorption				N	N
Tremor/twitching					N
Increased late activity				TC, TM	A, C, D, N, TC, TM

A, acetamiprid; C, clothianidin; D, dinotefuran; I, imidacloprid; N, nicotine; TC, thiacloprid; TM, thiamethoxam. ↓, reduced; ↓↓, severely reduced; ↓↓↓, not detectable; ↑, increased. K, kyphosis; L, lordosis. Areas shaded blue: time points during which this endpoint cannot be observed.

were observed. At 120 hpf, only increased motility in the 50 and 100 µM treatment groups was observed. At 120 hpf, dinotefuran exposure induced a trend similar to that of clothianidin. These observations were preceded by increased spontaneous movement (24 hpf) and pigmentation reduction (48 and 72 hpf) as well as spinal deformation and delayed hatching (72 hpf). Imidacloprid increased spontaneous movement at 24 hpf and induced morphological effects until 72 hpf, including circulatory reduction, reduced pigmentation, and spinal deformation. Embryos exposed to thiacloprid displayed a slight increase in motility at 24 and 96 hpf and an increased excitability upon agitation of the well-plates. Furthermore, reduced pigmentation (48 and 72 hpf) and

reduced heartbeat (72 hpf) were noted. Thiamethoxam-exposed embryos expressed increased spontaneous movement after 24 hpf as well as reduced circulation and pigmentation at 48 hpf. At 72 hpf, reduced hatching, spinal deformation, and reduced pigmentation were observed. At 96 hpf, hatching success was still reduced and spinal deformation was obvious. Increased activity was observed between 96 and 120 hpf.

3.3 Effects of neonicotinoids on behavior in embryonic zebrafish (coiling assay)

Nicotine-exposed zebrafish embryos showed a delayed onset of tail coiling, with the mean burst duration decreasing in a concentration-dependent manner (Fig. 1A). Before 37 hpf, exposure to > 1.25 µM induced significant alterations; however, after the onset of the second dark phase (37.5 hpf), all exposure concentrations induced a significant reduction in mean burst duration. Assessing the normalized mean burst duration (Fig. 1B), a clear concentration-dependent decrease is evident. The mean burst count per minute was further impaired by nicotine exposure (Fig. 1C), where 25 µM induced an immediate lack of burst initiations, whereas concentrations < 25 µM induced a concentration-dependent delay in the early burst count peak (around 24 hpf); the second peak at around 37 hpf was increased. Normalization to negative controls (0.1% DMSO) corroborated these trends: only 25 µM nicotine caused a persistent reduction in burst count, whereas exposure to < 25 µM induced a transient decrease followed by an increase (Fig. 1D).

Following thiacloprid exposure, the mean burst duration at later developmental stages was significantly reduced in a concentration-dependent manner, whereas early developmental stages were unaffected (Fig. 2A). Normalization to 0.1% DMSO confirmed the trend (Fig. 2B), since only exposure to < 100 µM induced short-term hyperactivity around 30 hpf. A non-significant reverse concentration-dependent hyperactivity after 26 hpf was observed for the burst counts per minute (Fig. 2C,D).

Exposure to 100 µM acetamiprid induced a significant reduction in the mean burst duration observed at 23 and 24 hpf, indicating a mild response to the onset of light (Fig. 3A). For the developmental phase between 37 and 47 hpf, a concentration-dependent reduction in burst duration was observed. Based on normalized data (Fig. 3B), the hyperactivity induced by acetamiprid as well as the subsequent hypoactivity became more evident. Effects of acetamiprid on burst counts per minute were less persistent; although statistical significance did not change from 23 and 24 hpf (Fig. 3C,D). In stages older than 38 hpf, exposure to 25 and 50 µM induced a significant increase in burst count (hyperactivity).

During early development, imidacloprid exposure significantly affected the burst duration behavior during light onset as well as causing a concentration-dependent decrease in late coiling behavior (Fig. 4A,B). The burst count per minute was less frequently significantly impacted by imidacloprid exposure. However, individuals also expressed a reduced response to the onset of light at 24 hpf. This is more clearly visible from the normalized data (Fig. 4C,D), as is the following hyperactivity in both analyzed parameters.

To further analyze zebrafish embryo behavior during the light/dark regime, changes induced in burst duration and burst count were computed from 37 to 38 hpf. For nicotine and thiacloprid (Fig. 5), all coiling assay replicates were utilized, whereas only one replicate (n = 1, 20 embryos per exposure group) was utilized for acetamiprid and imidacloprid (Fig. 6). Since the coiling assay data are given as mean values by automatic video analysis (Noldus software), standard deviation could be computed for 1 biological replicate (with 20 technical replicates). Thus, results presented in Figures 5 and 6 should be regarded as trend observations of zebrafish embryo behavior after exposure to neonicotinoids.

Nicotine exposure induced a trend towards the reduction of burst duration below the 10% threshold in all exposure groups (Fig. 5A), whilst more strongly affecting the burst frequency (Fig. 5B). Exposure to ≤ 25 µM induced burst count hyperactivity to beyond the +50% threshold (SC_{50}), whereas exposure to 25 µM strongly reduced burst counts. In contrast, thiacloprid exposure induced mild reduction in the burst duration to below 10% threshold (SC_{10}; Fig. 5C) and burst counts per minute increased in a concentration-dependent manner (Fig. 5D). These observations are based on trends in the data.

Acetamiprid and imidacloprid did not affect burst duration during the light change (Fig. 6A,C). Burst counts per minute, however, were increased following acetamiprid exposure, with individuals exposed to 25 and 100 µM surpassing the SC_{10}, while individuals exposed to 2.5 and 50 µM remained below the SC_{10} (Fig. 6B). Imidacloprid-exposed individuals initially showed a concentration-dependent increase in burst count per minute past the SC_{10} in response to the light change, reaching a plateau at ≥ 50 µM (Fig. 6D).

3.4 Effects of neonicotinoids on behavior in early larval stages of zebrafish (swimming assay)

In the swimming assay, no significant changes were observed in the movement distances or frequencies of zebrafish larvae exposed to ≤ 25 µM nicotine (Fig. 7A,C). However, upon normalization to the negative control, total distances moved were increased in a concentration-dependent fashion (Fig. 7B). Moreover, exposure to 1.25 µM nicotine induced an initial reduction in distance moved, followed by a transient activity peak and a subsequent reduction. In contrast, movement frequencies showed a transient reduction in all treatment groups (Fig. 7D), followed by an increase in frequency (i.e., similar to the behavior of untreated controls).

Individuals exposed to ≥ 50 µM acetamiprid displayed a reduction in total distance moved in comparison to the control group, whereas exposure to ≤ 25 µM initially led to an increase in distance moved (Fig. 8A). Normalization confirmed this trend, with only the 2.5 µM treatment group showing an increased distance moved throughout the swimming assay (Fig. 8B). Likewise, movement frequency was decreased upon exposure to ≤ 25 µM acetamiprid, whereas treatment with ≥ 50 µM acetamiprid returned movement frequencies to control levels or even slightly beyond controls (Fig. 8C). This trend was even more evident upon normalization (Fig. 8D).

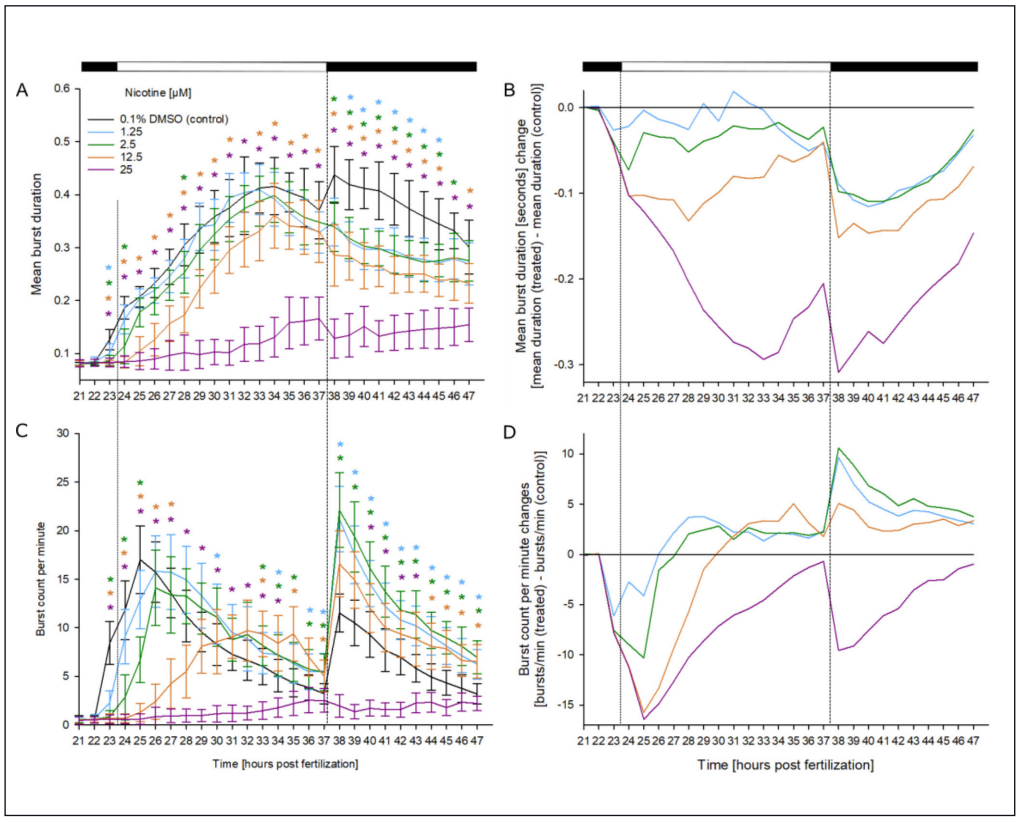

Fig. 1: Effects of nicotine on burst counts per minute and mean burst duration of zebrafish embryos during light/dark cycles in the coiling assay
Mean burst duration (sec; A) and normalized burst duration (B) as well as mean burst counts per minute (C) and normalized burst counts (D) between 21 and 47 h exposure to various concentrations of nicotine (n = 3; 20 embryos per concentration/replicate). Data given as mean ± standard deviation; B and D normalized to 0.1% DMSO. Top bars indicate phases of illumination. *p*-values of significant changes over DMSO controls can be found in Table S5[2].

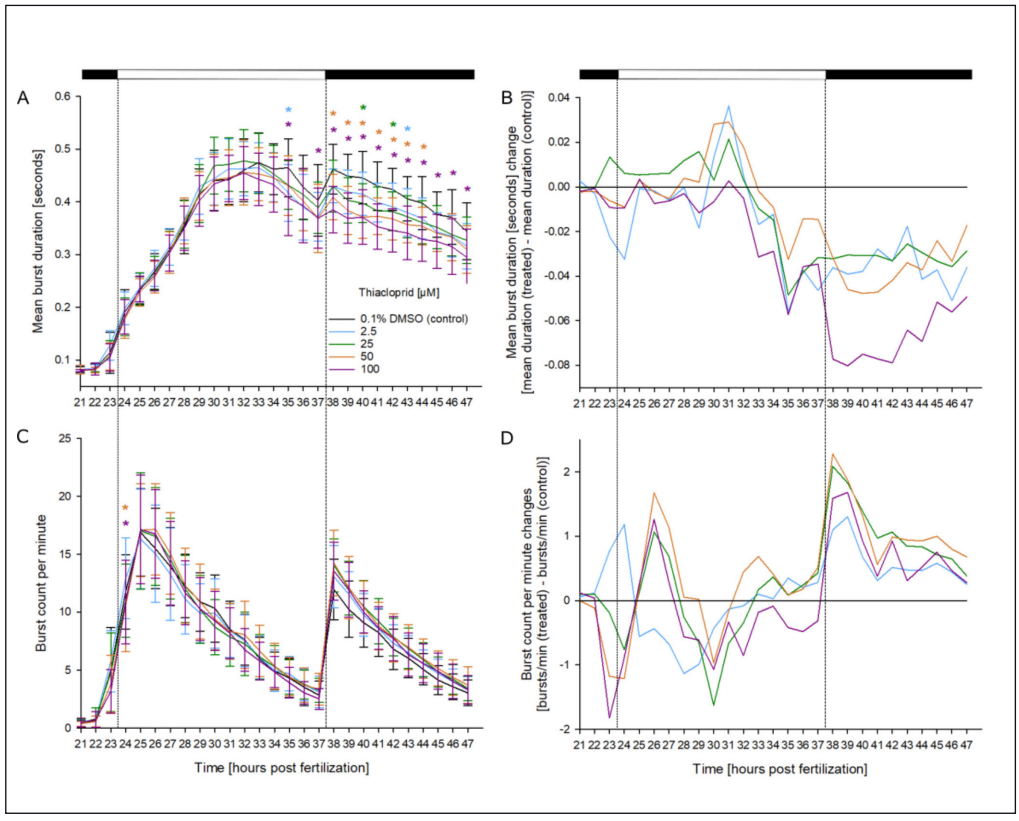

Fig. 2: Effects of thiacloprid on burst counts per minute and mean burst duration of zebrafish embryos during light/dark cycles in the coiling assay
Mean burst duration (sec; A) and normalized burst duration (B) as well as mean burst counts per minute (C) and normalized burst counts (D) between 21 and 47 h exposure to various concentrations of thiacloprid (n = 3; 20 embryos per concentration/replicate). Data given as mean ± standard deviation; B and D normalized to 0.1% DMSO. Top bars indicate phases of illumination. *p*-values of significant changes over DMSO controls can be found in Table S5[2].

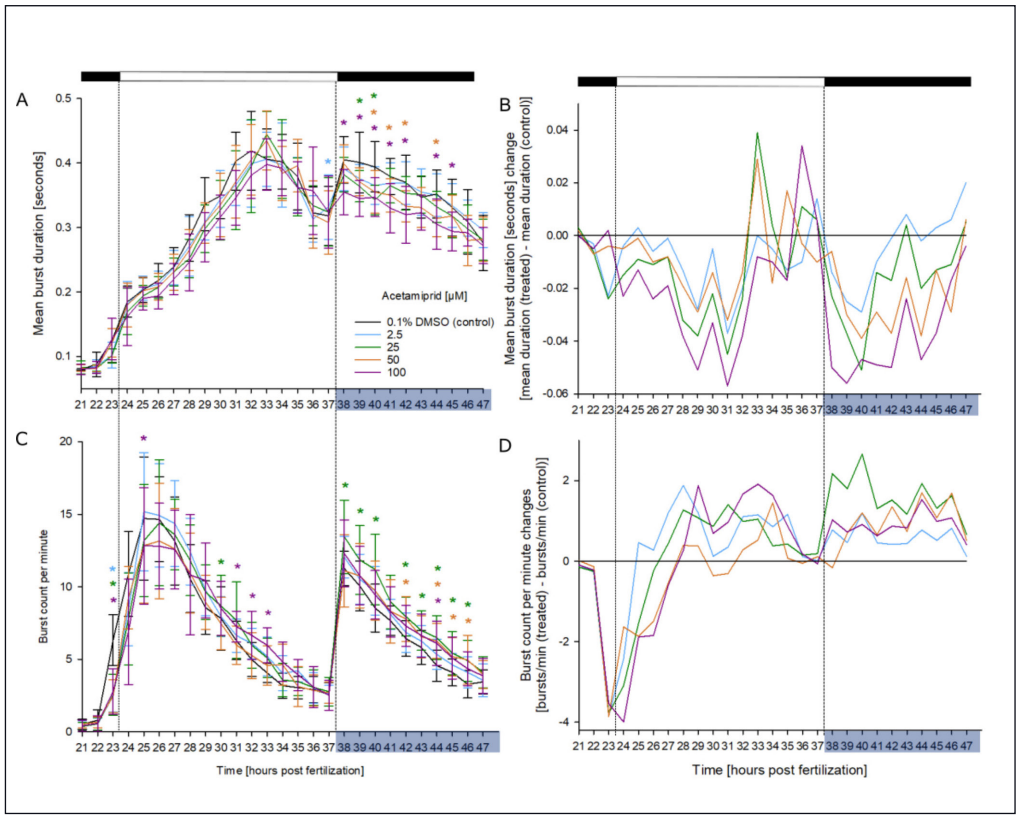

Fig. 3: Effects of acetamiprid on burst counts per minute and mean burst duration of zebrafish embryos during light/dark cycles in the coiling assay
Mean burst duration (sec; A) and normalized burst duration (B) as well as mean burst counts per minute (C) and normalized burst counts (D) between 21 and 47 h exposure to various concentrations of acetamiprid (n = 4 (21-37 hpf); n = 1 (38-47 hpf, indicated by the blue shading on the x-axis; 20 embryos per concentration/replicate). Data given as mean ± standard deviation; B and D normalized to 0.1% DMSO. Top bars indicate phases of illumination. *p*-Values of significant changes over DMSO controls can be found in Table S5[2].

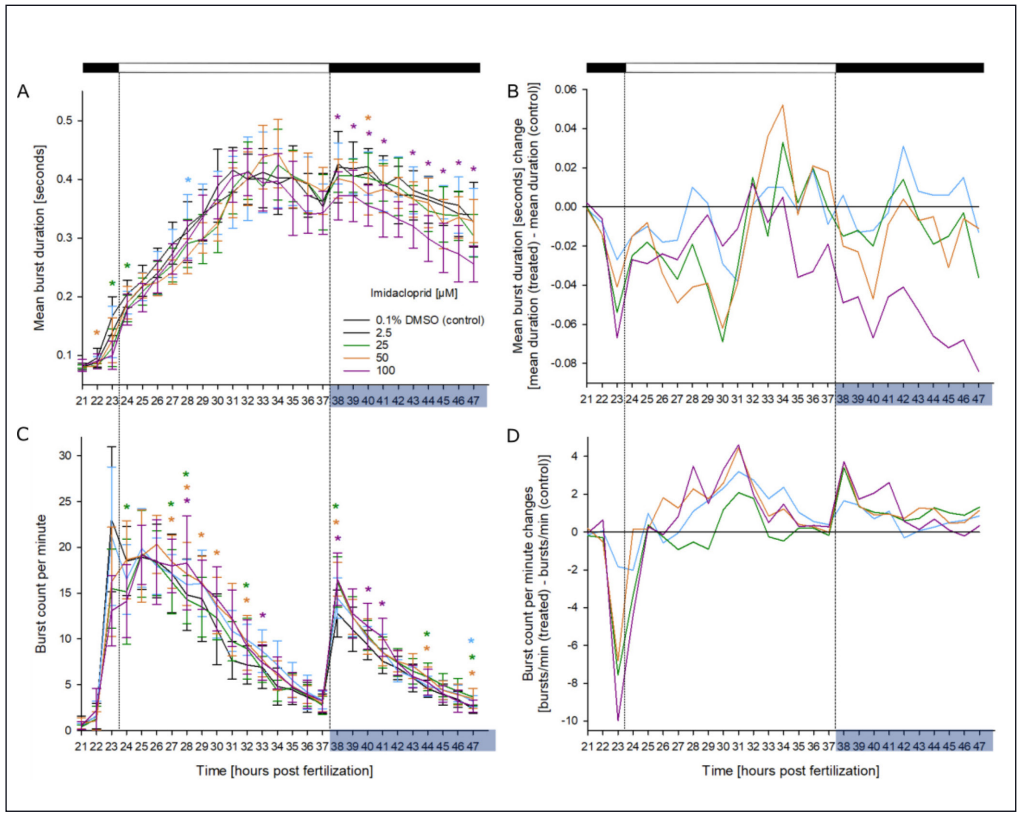

Fig. 4: Effects of imidacloprid on burst counts per minute and mean burst duration of zebrafish embryos during light/dark cycles in the coiling assay
Mean burst duration (sec; A) and normalized burst duration (B) as well as mean burst counts per minute (C) and normalized burst counts (D) between 21 and 47 h exposure to various concentrations of imidacloprid (n = 4 (21-37 hpf); n = 1 (38-47 hpf, indicated by the blue shading on the x-axis; 20 embryos per concentration/replicate). Data given as mean ± standard deviation; B and D normalized to 0.1% DMSO. Top bars indicate phases of illumination. *p*-Values of significant changes over DMSO controls can be found in Table S5[2].

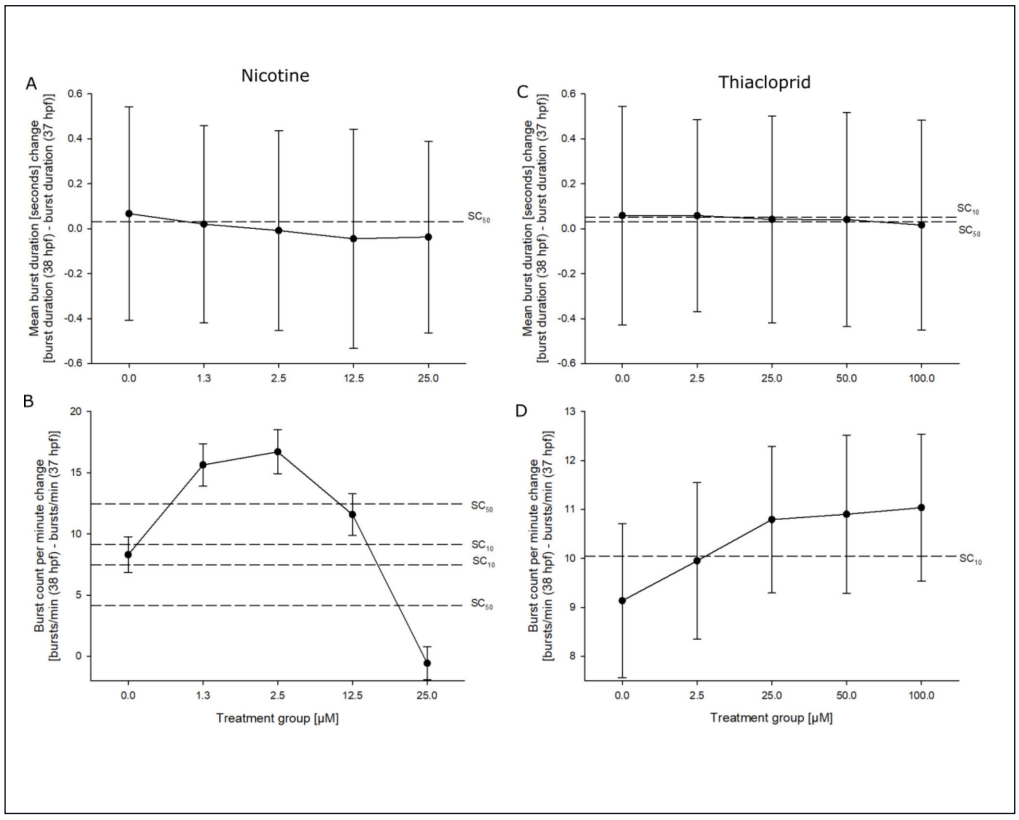

Fig. 5: Effects of nicotine and thiacloprid on burst counts per minute and mean burst duration of zebrafish embryos during change at 37.5 hpf in the coiling assay
Mean burst duration (sec; A) and burst count per minute (B) after exposure to various nicotine concentrations as well as the mean burst duration (sec; C) and burst count per minute (D) change after thiacloprid exposure (n = 3, 20 embryos per concentration). Data given as difference between the two time points ± standard deviation. SC_{10} and SC_{50} indicate thresholds for 10 and 50% deviation from the solvent control group, respectively. This presents a data trend but not a statistically significant difference between the exposure groups.

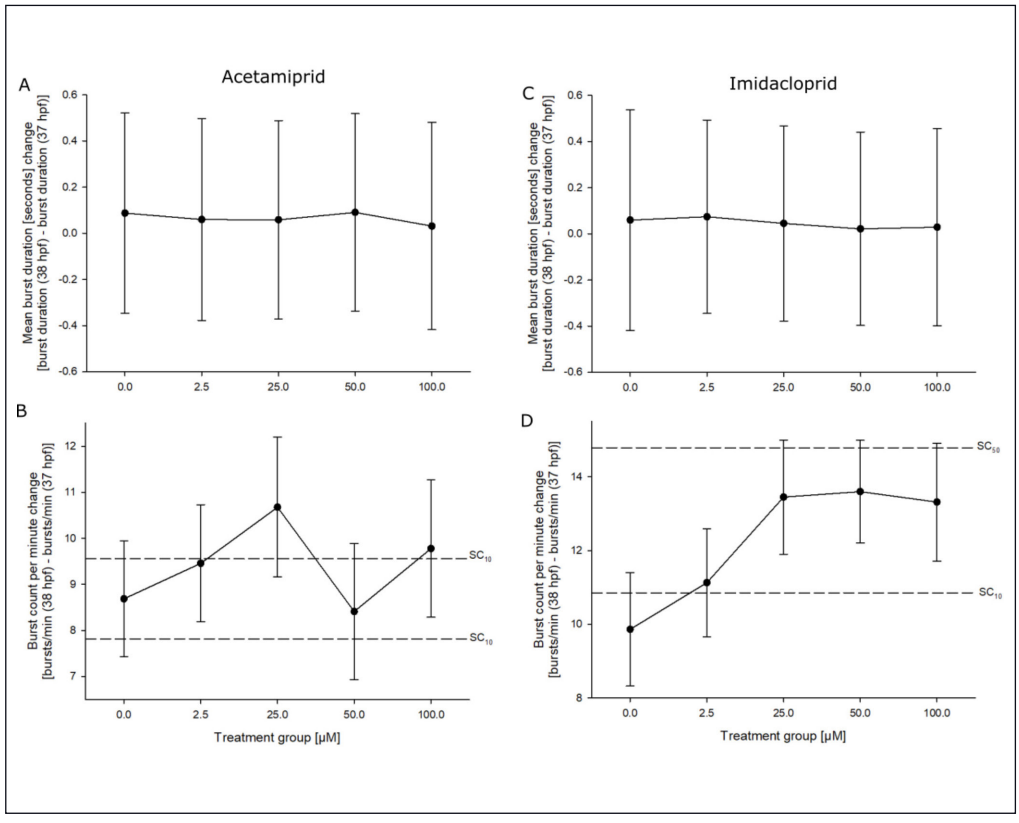

Fig. 6: Effects of acetamiprid and imidacloprid on burst counts per minute and mean burst duration of zebrafish embryos during change at 37.5 hpf in the coiling assay
Mean burst duration (sec; A) and burst count per minute change (B) after exposure to various acetamiprid concentrations, as well as the mean burst duration (sec; C) and burst count per minute change (D) after imidacloprid exposure (n = 1, 20 embryos per concentration). Data given as difference between the two time points ± standard deviation. SC_{10} and SC_{50} indicate thresholds for 10 and 50 % deviation from the solvent control group, respectively. This presents a data trend but not a statistically significant difference between the exposure groups.

The total distance moved was analyzed statistically over the entire swimming assay duration: Acetamiprid exposure decreased the distance moved with increasing concentration; exposure to ≥ 50 µM acetamiprid, however, led to a statistically significant reduction in movement distance (Fig. 9A). Nicotine exposure increased the distance moved in a concentration-dependent fashion (statistically significant from > 2.5 µM; Fig. 9B).

4 Discussion

The present study provided insight into the low acute toxicity of the tested neonicotinoids to zebrafish embryos, with compounds such as clothianidin, dinotefuran, and imidacloprid not reaching an EC_{50} with exposure concentrations as high as 100 µM. Previous FET data have only been published for acetamiprid, imidacloprid, nicotine, and thiacloprid, of which not all were concurrent with the data presented here and are discussed in more detail below. For the remaining neonicotinoids, no published FET test data could be obtained.

Whilst the determination of (D)NT in the zebrafish has found its application in ecotoxicological studies (e.g., Bambino and Chu, 2017; Galloway et al., 2017), its use for human risk assessment has only recently become a topic of interest. The use of behavioral changes as an endpoint for potential neurotoxic damage has been widely accepted, as it allows for the determination of whether, e.g., cellular damage has functional consequences for an individual (Vorhees et al., 2021). Given that, at least in the European Union, zebrafish embryos are not regarded as protected life-stages in the sense of the current animal welfare regulation (EU, 2010; Strähle et al., 2012), the present study exclusively used protocols that can be applied to zebrafish developmental stages up to 120 hpf: (1) The coiling assay (21-47 hpf) records spontaneous swimming behavior in non-hatched zebrafish embryos (Saint-Amant and Drapeau, 1998; Selderslaghs et al., 2010, 2013; Richendrfer et al., 2014; Zindler et al., 2019a,b); it is thus indicative for neurotoxic effects in early to intermediate developmental stages of zebrafish development. (2) The swimming assay (83-120 hpf) records the free-swimming behavior in hatched zebrafish embryos (Zindler et al., 2020) and is thus indicative of effects at later stages of development. For more details on effects of the neonicotinoids investigated, please refer to Table S3[2]. Given that comparably little information is available regarding the effects of neonicotinoids on fish, this publication aimed to provide a first report on the overall impact of these compounds on the neurodevelopment of zebrafish embryos using predefined behavioral assays. Although not providing detailed insight into effects on, e.g., specific receptors or indications of which (molecular or cellular) pathways might be disrupted through exposure, the macroscopically detectable behavioral alterations nonetheless provide insight into the compounds' potential toxicity and thus guide the way for potential future testing.

Since not all the compounds tested in this study were previously assessed, this publication provides an overview of existing *in vivo* data where present. Comparing mammalian assays with fish-based experiments comes with many challenges, including the different dosing regimens, exposure routes, statistical analysis, and experimental set-ups. Assessing the observed endpoints, however, it becomes evident that the tested neonicotinoids induce similar types of effects in the different test systems. With mammalian behavior being better understood (Bayne et al., 2015) and zebrafish behavior having received increasing attention in recent years (Levin and Cerutti, 2009), parallels can be drawn between the systems.

4.1 Medium sample analysis

Considering the medium analysis, it should be considered that repeated changes in temperature, especially freeze-thawing cycles, may affect the stability of neonicotinoids (Kozikowski et al., 2003). Although measures were taken to avoid repeated freeze-thawing cycles of the stock solution, handling of defrosted stock samples could not be avoided during daily handling. All medium samples collected were frozen in liquid nitrogen and transferred to -80°C for storage before being shipped on dry ice, thus leading again to variable temperatures. Although in the FET and behavioral test protocols standardized measures were taken to prevent evaporation (sealing of plates in the FET test, standardizing high humidity in the coiling and swimming assay test chambers), some evaporation cannot be ruled out. The aspect of solubility and potential influence of this parameter on the behavior and accumulation of each compound is addressed in the respective section of the discussion in more detail. This includes considerations that the hydrophobic nature of the neonicotinoids may, in part, be responsible for the reduced number and severity of effects observed in the assays conducted in the present study.

4.2 Nicotine

The FET test conducted in the present study determined the 96 hpf EC_{50} value of nicotine to be 23.5 µM. Similarly, Yoo et al. (2018) determined a 72 hpf LC_{44} of 40 µM, which corresponds with the 96 hpf EC_{50} value of the present study (44.7 µM). In the presented FET test, nicotine induced developmental alterations including spinal deformations such as lordosis and kyphosis, as well as enlarged pericardial volume. Previous studies with juvenile zebrafish also observed spinal deformation in conjunction with a reduction in overall body length after a 10-d exposure to nicotine (Parker and Connaughton, 2007). This is in line with the hypothesis that there is a direct link between spinal motoneurons and somite development (Wilt and Hake, 2003), meaning that compounds affecting the pathfinding ability of spinal motoneurons might also have an impact on the formation of musculature and overall physiology (Svoboda et al., 2002). Previous studies conducted with zebrafish also documented cardiovascular alterations (Yoo et al., 2018), linking heart deformation, edema, and reduced circulation to delayed transition of stem cells through the mesoderm (Palpant et al., 2015), with malignant arrhythmia and an abnormal cardiac conduction system linked to prenatal nicotine exposure in humans (Matturri et al., 2000), as well as in rats and sheep (Feng et al., 2010). The tremor observed in the present FET study might be related to the well-documented effect on nicotinic acetylcholine receptors (nAChRs), which are already present during embryogenesis and are vital for early neural

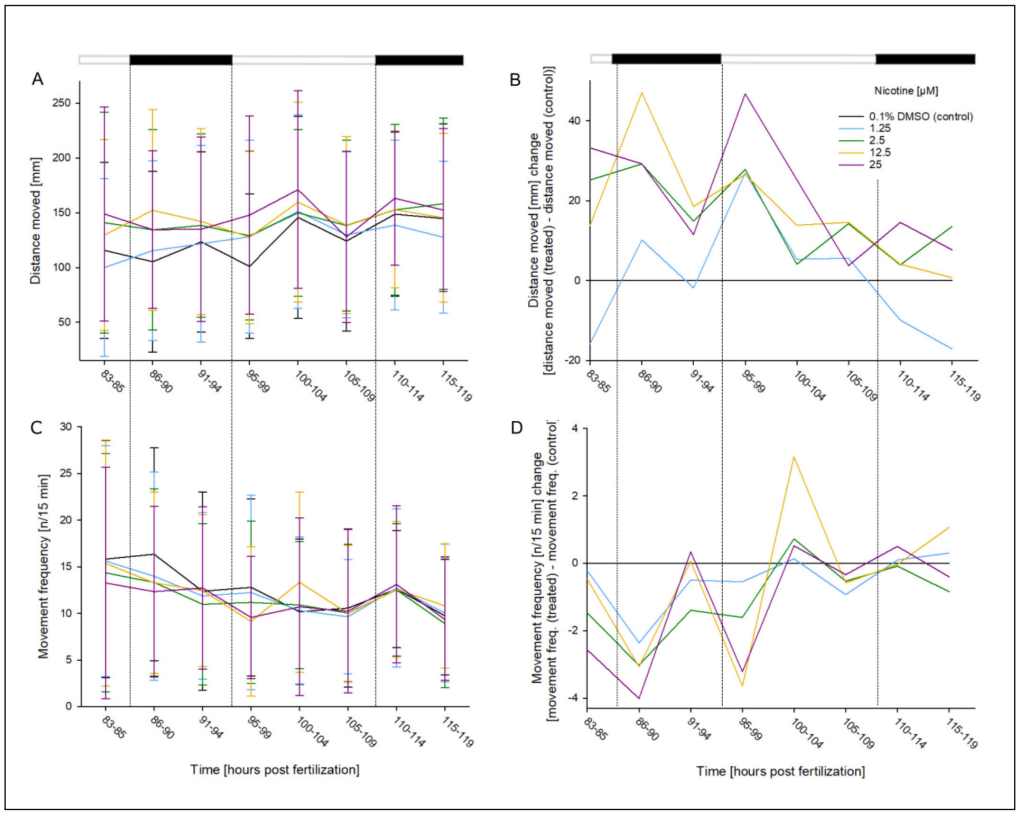

Fig. 7: Effects of nicotine on total distances moved and movement frequencies of zebrafish embryos during light/dark cycles in the swimming assay
The grouped total distance moved (min; A) and normalized grouped distance moved (B) as well as grouped frequencies of movement (n/15min; C) and normalized grouped frequency moved (D) between 83 and 120 hpf after exposure to various concentrations of nicotine (n = 3, 19 embryos per concentration/replicate). Data given as means ± standard deviation; B and D normalized to 0.1% DMSO. Top bars indicate phases of illumination

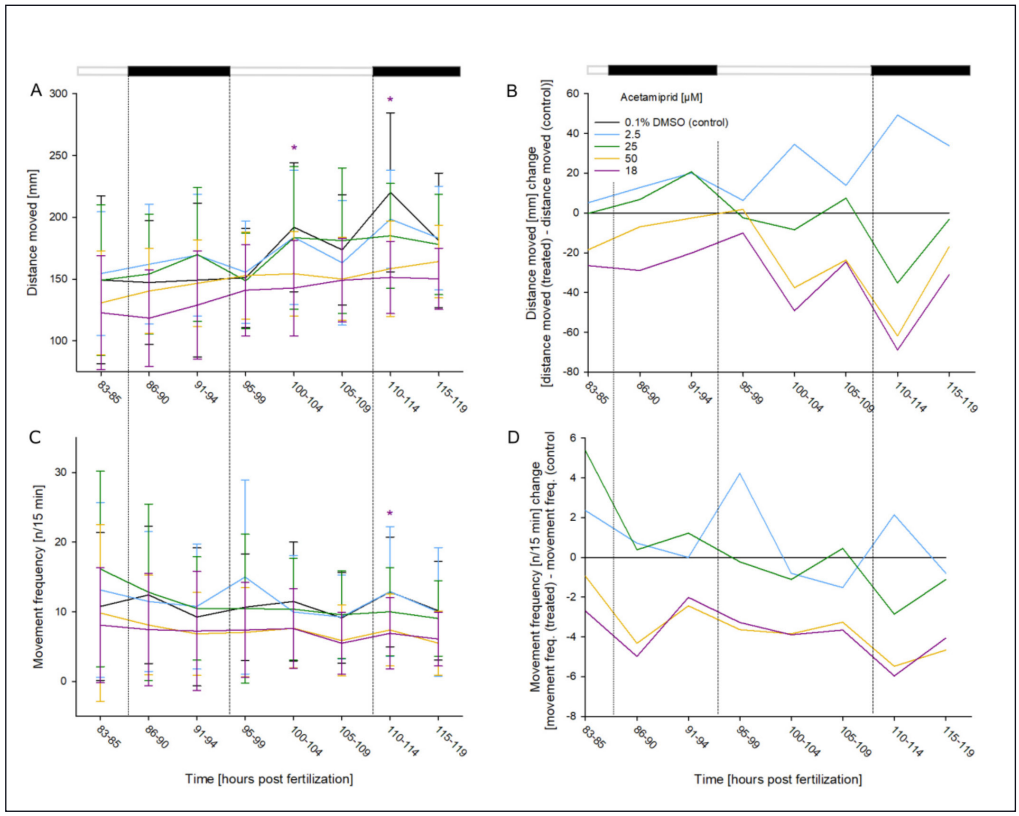

Fig. 8: Effects of acetamiprid on the total distance moved and movement frequency of zebrafish embryos during light/dark cycles in the swimming assay
The grouped total distance moved (min; A) and normalized grouped distance moved (B), as well as grouped frequencies of movement (n/15min; C) and normalized grouped frequency moved (D) between 83 and 120 hpf after exposure to various concentrations of acetamiprid (n = 2, 19 embryos per concentration/replicate). Data given as means ± standard deviation; B and D normalized to 0.1 % DMSO. Top bars indicate phases of illumination. p-Values of significant changes over DMSO controls can be found in Table S6[2].

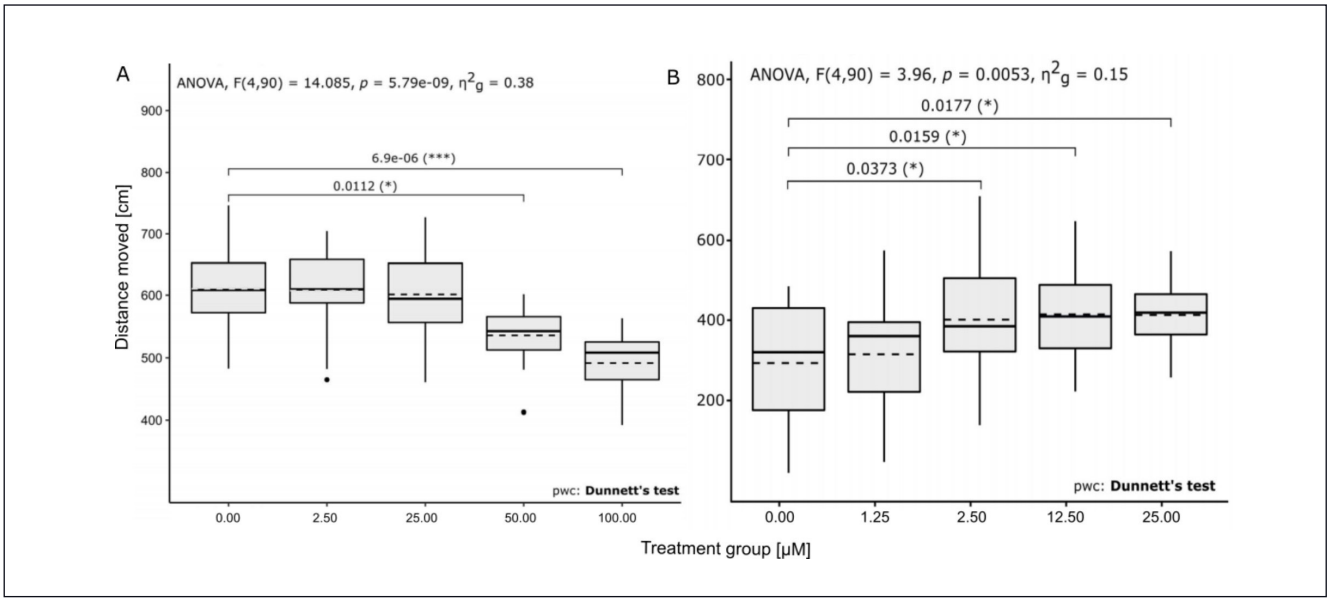

Fig. 9: Total distance moved (cm) from 83 to 120 hpf by zebrafish embryos exposed to acetamiprid (A) and nicotine (B) in the swimming assay

An ANOVA was conducted on the total distance moved per treatment group (N = 19/treatment group/replicate) and *post-hoc* pair-wise comparison (Dunnett's test) was run against the "0.00" treatment group (control group). The actual value was estimated as the mean square of each independent variable divided by the mean square of residuals. η^2g: generalized eta squared as an indicator for the strength of relationships between variables; solid lines: median; dashed lines: mean; *, $p < 0.05$; ***, $p < 0.0001$. *p*-Values are given in Table S7[2].

plate development (Svoboda et al., 2002). Finally, the craniofacial deformations seen in zebrafish embryos might well be related to the occurrence of cleft palates observed in offspring of mice dosed during gestation (Nishimura and Nakai, 1958), linking it to early neurodevelopmental disruptions.

Nicotine has been shown to cause concentration-dependent behavioral effects in zebrafish embryos (Ali et al., 2012), and the findings presented here are in line with these previous observations of studies showing paralysis after exposure to 33 µM nicotine linked to the activation and subsequent desensitization of muscle-specific nAChRs (Svoboda et al., 2002). Elevated concentrations of nicotine have been hypothesized to result in muscle degeneration and myopathy (Leonard and Salpeter, 1979), which were also noted after prolonged nicotine exposure in 7 d old zebrafish embryos (Welsh et al., 2009), possibly based on a post-excitatory increase in muscular calcium levels (Engel et al., 1982, 2003; Gomez et al., 2002).

Whilst the swimming assay conducted here was less indicative, Crosby et al. (2015) found that exposure to > 40 µM nicotine significantly reduced the swimming activity observed during dark conditions of 144 h old zebrafish juveniles. Bencan and Levin (2008) determined increased zebrafish swimming after exposure to > 600 µM nicotine and linked these findings to the α7 and α4β2 nAChRs, which play a vital role not only in anxiolytic responses, but also in normal brain development (Papke et al., 2012). Thus, nicotine is able to directly interact with the central nervous system by altering the motoneuron axon pathway (Welsh et al., 2009). Adolescent zebrafish expressed increased startle responses and swimming activity after sub-lethal nicotine exposure, whereas high doses progressively desensitized them to stimuli with prolonged exposure, whilst adult zebrafish exposed to 45 or 60 µM nicotine had a significantly reduced diving (i.e., hiding) response (Crosby et al., 2015). Conversely, nicotine caused an increase in activity at low doses, but led to reduced distance moved and rearing (standing on the hind legs) with increasing doses in mice (Freeman et al., 1987) and rats (Fung and Lau, 1989). Thus, hyperactivity has been documented as a symptom of low-dose nicotine exposure in both zebrafish and mammalian systems, whereas elevated doses resulted in a progressive decrease of activity in either test system.

4.3 Acetamiprid

Acetamiprid is considered highly water soluble , which may indicate that it is less able to penetrate the chorion and thus reach the embryo prior to hatching (de Koning et al., 2015). This might further provide an explanation for the effects observed here, as the exposure to acetamiprid in the FET test and coiling assay were less conclusive. It has also been determined that acetamiprid degrades moderately rapidly under the present experimental conditions[4] (EC, 2019), and the discrepancies observed be-

[4] https://www3.epa.gov/pesticides/chem_search/reg_actions/registration/fs_PC-099050_15-Mar-02.pdf

tween nominal and actual concentrations measured in medium were found to be within acceptable ranges.

In the present study, the 120 hpf EC_{50} value for acetamiprid was 63.8 µM, being notably more acutely toxic that in a previous study, where the 120 hpf EC_{50} value was determined to be 1.4 mM (Ma et al., 2019). The concentration range for the FET test presented here was limited to ≤ 100 µM. Developmental alterations such as circulatory modulation, spinal distortion, and craniofacial deformation were noted at low frequencies (only a few individuals were affected). This is in line with a previous study presenting an 120 h LC_{50} value of > 2 mM, with severe spinal malformations and edema among other observations (Ma et al., 2019). With respect to effects in mammals, a prenatal repeated dose experiment documented cortical plate hypoplasia and decreased neurogenesis in mice after exposure to 5 mg/kg body weight acetamiprid from day 6 to 13 of embryonic development. Exposure of newborn mice to the same exposure concentration, however, was found to induce abnormal neuronal distribution (Kagawa and Nagao, 2018). Further effects recorded in mice were reduced nAChR expression in the olfactory bulb and midbrain (Terayama et al., 2016).

As observed in the present coiling assay, a recent study also showed that exposure of zebrafish embryos to acetamiprid induced a concentration-dependent decrease in early "number of movements/min", as well as slightly delayed development, with a complete lack of response occurring after treatment with 4.4 mM. Additionally, the touch response of embryos between 27 and 36 hpf was significantly reduced after exposure to ≥ 2.4 mM (Ma et al., 2019). Given the significant difference in the concentration range used in the present study and by Ma et al. (2019), it is interesting to note that the present study revealed similar, although less prominent effects. Moreover, whilst their study noted that the touch response was not a sensitive endpoint, the response to light changes in our study was found to induce a significant change in burst counts per minute. No studies could be found on acetamiprid effects in early behavior in mammalian test species.

In the swimming assay presented here, acetamiprid exposure led to reduced swimming distance and frequency in hatched embryos. Since effects changed with prolonged exposure, Osterauer and Köhler (2008) speculated about the ability of the chorion to prevent higher acetamiprid levels from penetration and thus contact with the embryo (see also Vignet et al., 2019). However, the relatively low molecular weight (MW) of 222.68 g/mol as well as the structure of the molecule (log P_{ow} 0.79) make an important effect of the chorion highly unlikely (Henn and Braunbeck, 2011). In fact, 8 d old zebrafish exposed to environmentally relevant concentrations of acetamiprid showed a significant reduction in startle response habituation, whereas higher concentrations led to further reductions in habituation but an increase in the startle response itself (Faria et al., 2020). F1 male mice exposed to acetamiprid showed significantly reduced anxiety levels, whereas low-dose exposed adult males further expressed an increase in sexual and aggressive behavior (Sano et al., 2016). Thus, the zebrafish embryo presented effects seen in other studies conducted at higher concentrations. The reduction seen in movement frequency and distance in the swimming assay is thus likely to be a good indicator of a general decline in activity after exposure to acetamiprid.

4.4 Imidacloprid

The low measured concentrations of imidacloprid in the medium samples can be linked to photolysis rates of < 1 d, with degradation rates being increased at higher oxygen saturation (EC, 2011). The stability of imidacloprid is under debate, since there are reports that imidacloprid in water samples at environmentally relevant pH is stable (Yoshida, 1989), whereas other authors concluded rapid degradation by photolysis (Hellpointner, 1989; Krohn and Hellpointner, 2002). Additionally, similar to acetamiprid, imidacloprid is a pesticide of low lipophilicity (Gervais et al., 2012), which may affect its ability to cross the chorion in unhatched zebrafish embryos.

In the presented FET test, imidacloprid did not induce any effects after 48 hpf. Effects induced by imidacloprid in the present work included circulatory disturbances, delayed hatching, spinal deformation, and reduced pigmentation; changes, however, were only present until 72 hpf. In the only two publications assessing the compound in the FET test, however, imidacloprid exposure induced a 96 hpf LC_{50} between 475 and 562 µM (Wang et al., 2017; Wu et al., 2018). A potential explanation for the difference in observations may be the limited concentration range in the present publication. In another study, no mortality or any developmental or behavioral anomalies were seen at imidacloprid concentrations up to 7.8 mM (Vignet et al., 2019). The latter authors also considered the chorion to be a strong barrier during the first two days of exposure (MW 255.66 g/mol; log P_{ow} 0.57; barrier function of the chorion not likely; Henn and Braunbeck, 2011). In chicken embryos, heart tube malformation was observed after exposure during early development (Gao et al., 2016), which can be considered as an analogy to what was seen in the present FET study.

In the coiling assay, exposure to 100 µM imidacloprid induced a decrease in burst duration and an increase in burst counts per minute at a later stage of coiling behavior development. This is only the second time imidacloprid was studied in the zebrafish coiling assay (Ogungbemi et al., 2020); similar behavioral changes were observed in a study conducted with nicotine, thus similar explanations may be assumed (Leonard and Salpeter, 1979; Welsh et al., 2009). In hatched zebrafish embryos, exposure to > 25 µM imidacloprid reduced the distance moved after external stimuli such as tapping (Faria et al., 2019), which is again in line with the trends observed in the present study. It was found that imidacloprid impaired cognitive functions in rats, shown by a decline in learning abilities in both infants and adults after exposure to 2 and 8 mg/kg, respectively (Kara et al., 2015). In young adult offspring of mice dosed with 0.5 mg/kg/day during pre- and postnatal development, motor activity and social dominance was elevated, for example. Moreover, in an open field test, the total distance moved was increased significantly (Burke et al., 2018). Similar behavioral observations were previously made by Sheets (1994), who also highlighted a reduction in absolute brain weight after necropsy. Together, these findings in

mammals further support the relevance and sensitivity of behavioral alterations observed in the coiling assay with zebrafish embryos.

4.5 Thiacloprid

To date, there is no explanation for the > 100% actual concentrations of thiacloprid and thiamethoxam in the 50 and 100 µM nominal concentration samples; the three lower exposure samples followed the trend observed for the other neonicotinoids. Based on its high hydrophilicity, older publications explained the overall lack of effects by thiacloprid exposure in zebrafish embryos by the assumed barrier function of the lipophilic zebrafish chorion preventing substantial uptake (EC, 2019), although such barrier function had never been proven (Henn and Braunbeck, 2011).

The only previous study assessing thiacloprid in the zebrafish FET test determined a 96 hpf LC_{50} of 1.4 nM (Wang et al., 2020), thus being more acutely toxic than in the present study (96 hpf LC_{50}: > 200 mM). Embryos exposed to thiacloprid in the present study were more active in both early and late developmental stages, whereas only few morphological alterations were observed during the FET test. Osterauer and Köhler (2008) observed an increase in heartbeat rate followed by a reduction of heartbeat at higher concentrations in zebrafish embryo exposure to < 40 µM thiacloprid, but no developmental deformations were noted and hatching was not affected. The increased heartbeat in thiacloprid-exposed zebrafish embryos observed in the present study might provide an explanation for the increase in burst duration and unaffected burst counts.

Studies on effects of thiacloprid on embryonic development in mammals are scarce. One single study reported reduced blastomeres and a higher percentage of dead blastocysts in isolated mouse and rabbit embryos (Babeľová et al., 2017). Both in fish and mammals, more studies are needed for an in-depth understanding of effects by thiacloprid during vertebrate development.

4.6 Clothianidin

The overall very low actual concentrations of clothianidin samples may be explained by accelerated photolysis rates (half-life time of 3.3 h at 25°C) leading to essentially non-existence after handling (FAO, 2016). Moreover, the lack of observations in comparison with nicotine may again be due to the more hydrophilic properties of clothianidin in comparison to nicotine[5].

The present study is the first to report effects of clothianidin on zebrafish embryos in the FET test. However, only minor morphological effects in zebrafish embryos exposed to clothianidin for 48 and 96 h were observed. With respect to behavioral effects, clothianidin exposure for 120 h stimulated motor activity at low exposure concentrations, but reduced activity at higher concentrations. This may be due, in part, to the photolytic degradation and subsequently very low clothianidin levels found in the exposure medium (FAO, 2016) or relate to the nAChR desensitizing effects of the neonicotinoids in general (Giniatullin et al., 2005). In the literature, there is just one study on the startle response of zebrafish at an age of 8 d, which observed a significant increase in response to external stimuli, however, above environmental concentrations (Faria et al., 2020).

In pregnant mice, clothianidin was rapidly transferred to the fetuses (Ohno et al., 2020), and isolated mouse embryos showed reduced embryonic development (Babeľová et al., 2017). Behavioral studies revealed significant alterations in both adult and juvenile mouse behavior after dietary uptake of clothianidin (Tanaka, 2012). Single dosing of male mice led to increased anxiety in the elevated plus-maze test at concentrations below the no observable adverse effect level (NOAEL) (Hirano et al., 2018). In rats, clothianidin had no effect on memory and learning abilities (Özdemir et al., 2014).

4.7 Dinotefuran

Dinotefuran has been determined to degrade only moderately rapidly under the present experimental conditions[4] (EC, 2019), and the discrepancies between nominal and actual concentrations observed in the analyzed medium samples were determined to be within acceptable ranges. The overall lack of observations in early developmental stages of zebrafish embryo assays may be influenced by its hydrophilicity[6], as outlined for the previously discussed neonicotinoids.

During later stages of development, dinotefuran exposure induced similar effects as clothianidin; however, these were already evident from 24 h. Since nominal and actual concentrations did not differ significantly, dinotefuran may thus be assumed to not produce severe effects in zebrafish embryos. Whereas there are no data on acute toxicity of dinotefuran in zebrafish embryos, a study into the startle response in 8 d old zebrafish exposed to environmentally relevant concentrations showed a significant reduction of the habituation response to external stimuli (Faria et al., 2020). In juvenile Chinese rare minnow (*Gobiocypris rarus*), exposure to 2.5 µM dinotefuran affected antioxidant enzymes along with various gene transcription levels (Tian et al., 2020).

Again, data for effects by dinotefuran in mammals are scarce. Isolated mouse embryos showed reduced embryonic development after prenatal exposure (Babeľová et al., 2017), while male mice showed a dose-dependent increase in activity in open field experiments (Yoneda et al., 2018) but no depression-like symptoms, as previously assumed (Takada et al., 2018).

4.8 Thiamethoxam

To date, there is no explanation for the > 100% actual concentrations of thiamethoxam in the 50 and 100 µM nominal concentration samples; the three lower exposure samples followed the trend observed for the other neonicotinoids. Thiamethoxam, like the remaining neonicotinoids, is hydrophilic, which is likely

[5] https://www.federalregister.gov/documents/2009/12/09/E9-29339/clothianidin-pesticide-tolerances

[6] https://www3.epa.gov/pesticides/chem_search/reg_actions/registration/fs_PC-044312_01-Sep-04.pdf

to affect its ability to cross the chorion and will thus likely influence the exposure during early developmental stages of the zebrafish embryo.

Whereas thiamethoxam induced only few early developmental changes, 120 h exposure resulted in obvious changes of activity in the FET test. One study determined that exposure to 292 µM thiamethoxam did not affect 48 h survival and subsequent hatching, overall growth, eye and head size as well as head-body angle; even chorion surface tension was unaffected by the exposure to thiamethoxam (Liu et al., 2018). In isolated mouse embryos, there is only one study reporting a negative impact of thiamethoxam on development (Babeľová et al., 2017). Thus, there is an urgent need for additional studies on thiamethoxam-related effects in both fish and mammalian development.

5 Conclusions

This study aimed to determine the suitability of the two selected behavioral assays to identify the DNT potential of neonicotinoids in zebrafish embryos at different developmental stages. The data presented clearly document that exposure to the neonicotinoids induced changes in both coiling and swimming behavior at sub-lethal exposure concentrations. Here, the chemical properties of the compounds need to be taken into account, as lipophilicity and molecule size have an impact on their ability to cross the chorion in early developmental stages and will thus have a profound influence on the exposure scenario.

The complexity of the human nervous system, especially the brain, has remained a major challenge when it comes to the determination of the site(s) affected by a toxicant and to the identification of processes leading to (D)NT. Especially when the outcome is unknown, such studies have most frequently been conducted *in vivo*. However, in light of the quest for the refinement, reduction, and replacement of animal tests (Russell and Burch, 1959), alternative test systems like zebrafish embryos are highly sought after. They allow for a compromise combining *in vivo* complexity whilst addressing the ethical questions linked to animal studies. Especially in combination with behavioral assays, the present study has successfully documented the capacity of the fish embryo test (FET) to detect the (D)NT potential of neonicotinoids. Both the coiling (21-47 hpf) and swimming assay (83-120 hpf) revealed behavioral alterations that could be interpreted as indicative of observations in mammalian studies. Following a "positive hit" in these indicator assays, modified exposure regimes or external stimuli might be introduced to discriminate the pathway(s) affected and understand the toxic potential of the compound further. Such findings could then be supported by targeted *in vitro* analyses as well as *in silico* modelling, thus forming an alternative (D)NT test battery of new approach methods (Masjosthusmann et al., 2020; Loser et al., 2021). However, for these assays to become generally acceptable, standardization of protocols, fine-tuning of data analysis, and more mechanism-based research in the field of DNT at sub-lethal concentrations should be next steps. Although the FET test has been challenged to be insensitive to neurotoxicants (Sobanska et al., 2018), the present study clearly documents that implementation of additional endpoints makes this test a promising candidate component of a test battery suited for even high-throughput NT and DNT research.

References

Ali, S., Champagne, D. L. and Richardson, M. K. (2012). Behavioral profiling of zebrafish embryos exposed to a panel of 60 water-soluble compounds. *Behav Brain Res 228*, 272-283. doi:10.1016/j.bbr.2011.11.020

Aschner, M., Ceccatelli, D., Daneshian, M. et al. (2017). Reference compounds for alternative test methods to indicate developmental neurotoxicity (DNT) potential of chemicals: Example lists and criteria for their selection and use. *ALTEX 34*, 49-73. doi:10.14573/altex.1604201

Auteri, D., Arena, M., Barmaz, S. et al. (2017). Neonicotinoids and bees: The case of the European regulatory risk assessment. *Sci Total Environ 579*, 966-971. doi:10.1016/j.scitotenv.2016.10.158

Babeľová, J., Šefčíková, Z., Čikoš, Š. et al. (2017). Exposure to neonicotinoid insecticides induces embryotoxicity in mice and rabbits. *Toxicology 392*, 71-80. doi:10.1016/j.tox.2017.10.011

Bailey, J., Thew, M. and Balls, M. (2014). An analysis of the use of animal models in predicting human toxicology and drug safety. *Altern Lab Anim 42*, 181-199. doi:10.1177/026119291404200306

Bambino, K. and Chu, J. (2017). Zebrafish in toxicology and environmental health. *Curr Top Dev Biol 124*, 331-367. doi:10.1016/bs.ctdb.2016.10.007

Basnet, R., Zizioli, D., Taweedet, S. et al. (2019). Zebrafish larvae as a behavioral model in neuropharmacology. *Biomedicines 7*, 23. doi:10.3390/biomedicines7010023

Bass, S. L. S. and Gerlai, R. (2008). Zebrafish (Danio rerio) responds differentially to stimulus fish: The effects of sympatric and allopatric predators and harmless fish. *Behav Brain Res 186*, 107-117. doi:10.1016/j.bbr.2007.07.037

Bayne, K. A. L., Beaver, B. V., Mench, J. A. et al. (2015). Chapter 38 – Laboratory animal behavior. In J. G. Fox, L. C. Anderson, G. M. Otto et al. (eds), *Laboratory Animal Medicine* (1617-1651). 3rd edition. Academic Press. doi:10.1016/B978-0-12-409527-4.00038-9

Bencan, Z. and Levin, E. D. (2008). The role of α7 and α4β2 nicotinic receptors in the nicotine-induced anxiolytic effect in zebrafish. *Physiol Behav 95*, 408-412. doi:10.1016/j.physbeh.2008.07.009

Braunbeck, T., Kais, B., Lammer, E. et al. (2015). The fish embryo test (FET): Origin, applications, and future. *Environ Sci Pollut Res Int 22*, 16247-16261. doi:10.1007/s11356-014-3814-7

Burke, A. P., Niibori, Y., Terayama, H. et al. (2018). Mammalian susceptibility to a neonicotinoid insecticide after fetal and early postnatal exposure. *Sci Rep 8*, 16639. doi:10.1038/s41598-018-35129-5

Chang, L. W. (1998). Introduction. In W. Slikker and L. W. Chang (eds.), *Handbook of Developmental Neurotoxicology* (1-2). Academic Press. doi:10.1016/B978-012648860-9.50003-0

Clark, M. and Steger-Hartmann, T. (2018). A big data approach to the concordance of the toxicity of pharmaceuticals in animals and humans. *Regul Toxicol Pharmacol 96*, 94-105. doi:10.1016/j.yrtph.2018.04.018

Crosby, E. B., Bailey, J. M., Oliveri, A. N. et al. (2015). Neurobehavioral impairments caused by developmental imidacloprid exposure in zebrafish. *Neurotoxicol Teratol 49*, 81-90. doi:10.1016/j.ntt.2015.04.006

Cunha-Oliveira, T., Rego, A. C. and Oliveira, C. R. (2008). Cellular and molecular mechanisms involved in the neurotoxicity of opioid and psychostimulant drugs. *Brain Res Rev 58*, 192-208. doi:10.1016/j.brainresrev.2008.03.002

de Koning, C., Beekhuijzen, M., Tobor-Kapłon, M. et al. (2015). Visualizing compound distribution during zebrafish embryo development: The effects of lipophilicity and DMSO. *Birth Defects Res Part B Dev Reprod Toxicol 104*, 253-272. doi:10.1002/bdrb.21166

Delp, J., Gutbier, S., Klima, S. et al. (2018). A high-throughput approach to identify specific neurotoxicants/developmental toxicants in human neuronal cell function assays. *ALTEX 35*, 235-253. doi:10.14573/altex.1712182

Drapeau, P., Saint-Amant, L., Buss, R. R. et al. (2002). Development of the locomotor network in zebrafish. *Prog Neurobiol 68*, 85-111. doi:10.1016/S0301-0082(02)00075-8

Dubovický, M., Kovačovský, P., Ujházy, E. et al. (2008). Evaluation of developmental neurotoxicity: Some important issues focused on neurobehavioral development. *Interdiscip Toxicol 1*, 206-210. doi:10.2478/v10102-010-0042-y

EC (2011). Directive 98/8/EC concerning the placing of biocidal products on the market. Imidacloprid Product-type 18 (Insecticides, Acaricides and Product to control other Arthropods). https://echa.europa.eu/documents/10162/225b9c58-e24c-6491-cc8d-7d85564f3912

EC (2019). Regulation (EU) No 528/2012 concerning the making available on the market and use of biocidal products. Dinotefuran Product-type 18 (Insecticides, acaricides and to control other arthropods). https://echa.europa.eu/documents/10162/6f1a5175-f1eb-4e6b-4b49-8cea13efcf5a

EFSA – European Food Safety Authority (2013). Scientific opinion on the developmental neurotoxicity potential of acetamiprid and imidacloprid. *EFSA J 11*, 3471. doi:10.2903/j.efsa.2013.3471

Engel, A. G., Lambert, E. H., Mulder, D. M. et al. (1982). A newly recognized congenital myasthenic syndrome attributed to a prolonged open time of the acetylcholine-induced ion channel. *Ann Neurol 11*, 553-569. doi:10.1002/ana.410110603

Engel, A. G., Ohno, K., Shen, X.-M. et al. (2003). Congenital myasthenic syndromes: Multiple molecular targets at the neuromuscular junction. *Ann N Y Acad Sci 998*, 138-160. doi:10.1196/annals.1254.016

EU – European Union (2010). Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. *OJ L 276, 33-79*. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32010L0063&qid=1648544293278&from=EN

FAO - Food and Agriculture Organization (2016). FAO Specifications and Evaluations for Agricultural Pesticides. Clothianidin. https://www.fao.org/3/ca7726en/ca7726en.pdf

Faria, M., Prats, E., Novoa-Luna, K. A. et al. (2019). Development of a vibrational startle response assay for screening environmental pollutants and drugs impairing predator avoidance. *Sci Total Environ 650*, 87-96. doi:10.1016/j.scitotenv.2018.08.421

Faria, M., Wu, X., Luja-Mondragón, M. et al. (2020). Screening anti-predator behaviour in fish larvae exposed to environmental pollutants. *Sci Total Environ 714*, 136759. doi:10.1016/j.scitotenv.2020.136759

Feng, Y., Caiping, M., Li, C. et al. (2010). Fetal and offspring arrhythmia following exposure to nicotine during pregnancy. *J Appl Toxicol 30*, 53-58. doi:10.1002/jat.1471

Franks, M. E., Macpherson, G. R. and Figg, W. D. (2004). Thalidomide. *Lancet 363*, 1802-1811. doi:10.1016/S0140-6736(04)16308-3

Freeman, G. B., Sherman, K. A. and Gibson, G. E. (1987). Locomotor activity as a predictor of times and dosages for studies of nicotine's neurochemical actions. *Pharmacol Biochem Behav 26*, 305-312. doi:10.1016/0091-3057(87)90123-7

Fritsche, E. (2017). OECD/EFSA workshop on developmental neurotoxicity (DNT): The use of non-animal test methods for regulatory purposes. *ALTEX 34*, 311-315. doi:10.14573/altex.1701171

Fung, Y. K. and Lau, Y. S. (1989). Effects of prenatal nicotine exposure on rat striatal dopaminergic and nicotinic systems. *Pharmacol Biochem Behav 33*, 1-6. doi:10.1016/0091-3057(89)90419-x

Galloway, T. S., Dogra, Y., Garrett, N. et al. (2017). Ecotoxicological assessment of nanoparticle-containing acrylic copolymer dispersions in fairy shrimp and zebrafish embryos. *Environ Sci Nano 4*, 1981-1997. doi:10.1039/C7EN00385D

Gao, L., Li, S., Zhang, J. et al. (2016). Excess imidacloprid exposure causes the heart tube malformation of chick embryos. *J Agric Food Chem 64*, 9078-9088. doi:10.1021/acs.jafc.6b03381

Gervais, J. A., Luukinen, B., Buhl, K. et al. (2012). Imidacloprid technical fact sheet. *Natl Pestic Inf Cent.* http://npic.orst.edu/factsheets/archive/imidacloprid.html

Giniatullin, R., Nistri, A. and Yakel, J. L. (2005). Desensitization of nicotinic ACh receptors: Shaping cholinergic signaling. *Trends Neurosci 28*, 371-378. doi:10.1016/j.tins.2005.04.009

Gomez, C. M., Maselli, R. A., Groshong, J. et al. (2002). Active calcium accumulation underlies severe weakness in a panel of mice with slow-channel syndrome. *J Neurosci 22*, 6447-6457. doi:10.1523/JNEUROSCI.22-15-06447.2002

Health & Chemicals (2002). Minamata Disease the History and Measures. http://www.env.go.jp/en/chemi/hs/minamata2002/

Hellpointner, E. (1990). Determination of the quantum yield and assessment of the environmental half-life of the direct photodegradation of imidacloprid in water. Report No. PF3422.

Henn, K. and Braunbeck, T. (2011). Dechorionation as a tool to improve the fish embryo toxicity test (FET) with the zebrafish (Danio rerio). *Comp Biochem Physiol C Toxicol Pharmacol 153*, 91-98. doi:10.1016/j.cbpc.2010.09.003

Hirano, T., Yanai, S., Takada, T. et al. (2018). NOAEL-dose of a neonicotinoid pesticide, clothianidin, acutely induce anxiety-related behavior with human-audible vocalizations in male mice in a novel environment. *Toxicol Lett 282*, 57-63. doi:10.1016/j.toxlet.2017.10.010

Hoelting, L., Klima, S., Karreman, C. et al. (2016). Stem cell-derived immature human dorsal root ganglia neurons to identify peripheral neurotoxicants. *Stem Cells Transl Med 5*, 476-487. doi:10.5966/sctm.2015-0108

Kagawa, N. and Nagao, T. (2018). Neurodevelopmental toxicity in the mouse neocortex following prenatal exposure to acetamiprid. *J Appl Toxicol 38*, 1521-1528. doi:10.1002/jat.3692

Kalueff, A. V., Gebhardt, M., Stewart, A. M. et al. (2013). Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. *Zebrafish 10*, 70-86. doi:10.1089/zeb.2012.0861

Kara, M., Yumrutas, O., Demir, C. F. et al. (2015). Insecticide imidacloprid influences cognitive functions and alters learning performance and related gene expression in a rat model. *Int J Exp Pathol 96*, 332-337. doi:10.1111/iep.12139

Kimmel, C. B., Patterson, J. and Kimmel, R. O. (1974). The development and behavioral characteristics of the startle response in the zebra fish. *Dev Psychobiol 7*, 47-60. doi:10.1002/dev.420070109

Kimmel, C., Ballard, W., Kimmel, S. et al. (1995). Stages of embryonic development of the zebrafish. *Am J Anat 203*, 253-310. doi:10.1002/aja.1002030302

Kitamura, S., Miyata, C., Tomita, M. et al. (2020). A central nervous system disease of unknown cause that occurred in the minamata region: Results of an epidemiological study. *J Epidemiol 30*, 3-11. doi:10.2188/jea.JE20190173

Klüver, N., König, M., Ortmann, J. et al. (2015). Fish embryo toxicity test: Identification of compounds with weak toxicity and analysis of behavioral effects to improve prediction of acute toxicity for neurotoxic compounds. *Environ Sci Technol 49*, 7002-7011. doi:10.1021/acs.est.5b01910

Kokel, D., Bryan, J., Laggner, C. et al. (2010). Rapid behavior-based identification of neuroactive small molecules in the zebrafish. *Nat Chem Biol 6*, 231-237. doi:10.1038/nchembio.307

Kozikowski, B. A., Burt, T. M., Tirey, D. A. et al. (2003). The effect of freeze/thaw cycles on the stability of compounds in DMSO. *J Biomol Screen 8*, 210-215. doi:10.1177/1087057103252618

Krohn, J. and Hellpointner, E. (2002). Environmental fate of imidacloprid. *Pflanzenschutz Nachrichten Bayer 55*, 1-25. http://cues.cfans.umn.edu/old/pollinators/pdf-pesticides/2006CAImidclprdfate.pdf

Lammer, E., Carr, G. J., Wendler, K. et al. (2009). Is the fish embryo toxicity test (FET) with the zebrafish (Danio rerio) a potential alternative for the fish acute toxicity test? *Comp Biochem Physiol C Toxicol Pharmacol 149*, 196-209. doi:10.1016/j.cbpc.2008.11.006

Leonard, J. P. and Salpeter, M. M. (1979). Agonist-induced myopathy at the neuromuscular junction is mediated by calcium. *J Cell Biol 82*, 811-819. doi:10.1083/jcb.82.3.811

Letcher, R. J., Bustnes, J. O., Dietz, R. et al. (2010). Exposure and effects assessment of persistent organohalogen contaminants in arctic wildlife and fish. *Sci Total Environ 408*, 2995-3043. doi:10.1016/j.scitotenv.2009.10.038

Levin, E. D. and Cerutti, D. T. (2009). Behavioral neuroscience of zebrafish. In J. J. Buccafusco (ed.), *Methods of Behavior Analysis in Neuroscience* (Chapter 15). 2nd edition. CRC Press/Taylor & Francis.

Liu, X., Zhang, Q., Li, S. et al. (2018). Developmental toxicity and neurotoxicity of synthetic organic insecticides in zebrafish (Danio rerio): A comparative study of deltamethrin, acephate, and thiamethoxam. *Chemosphere 199*, 16-25. doi:10.1016/j.chemosphere.2018.01.176

Loser, D., Hinojosa, M. G., Blum, J. et al. (2021). Functional alterations by a subgroup of neonicotinoid pesticides in human dopaminergic neurons. *Arch Toxicol 95*, 2081-2107. doi:10.1007/s00204-021-03031-1

Ma, X., Li, H., Xiong, J. et al. (2019). Developmental toxicity of a neonicotinoid insecticide, acetamiprid to zebrafish embryos. *J Agric Food Chem 67*, 2429-2436. doi:10.1021/acs.jafc.8b05373

Masjosthusmann, S., Blum, J., Bartmann, K. et al. (2020). Establishment of an a priori protocol for the implementation and interpretation of an in-vitro testing battery for the assessment of developmental neurotoxicity. *EFSA Support Publ 17*, 1938E. doi:10.2903/sp.efsa.2020.EN-1938

Matturri, L., Ottaviani, G., Ramos, S. G. et al. (2000). Sudden infant death syndrome (SIDS): A study of cardiac conduction system. *Cardiovasc Pathol 9*, 137-145. doi:10.1016/S1054-8807(00)00035-1

Millot, F., Decors, A., Mastain, O. et al. (2017). Field evidence of bird poisonings by imidacloprid-treated seeds: A review of incidents reported by the French SAGIR network from 1995 to 2014. *Environ Sci Pollut Res 24*, 5469-5485. doi:10.1007/s11356-016-8272-y

Monticello, T. M., Jones, T. W., Dambach, D. M. et al. (2017). Current nonclinical testing paradigm enables safe entry to first-in-human clinical trials: The IQ consortium nonclinical to clinical translational database. *Toxicol Appl Pharmacol 334*, 100-109. doi:10.1016/j.taap.2017.09.006

Nishimura, H. and Nakai, K. (1958). Developmental anomalies in offspring of pregnant mice treated with nicotine. *Science 127*, 877-878. doi:10.1126/science.127.3303.877

OECD (2007). Test No. 426: Developmental Neurotoxicity Study. *OECD Guidelines for the Testing of Chemicals, Section 4*. OECD Publishing, Paris. doi:10.1787/9789264067394-en

OECD (2013). Test No. 236: Fish Embryo Acute Toxicity (FET) Test. *OECD Guidelines for the Testing of Chemicals, Section 2*. OECD Publishing, Paris. doi:10.1787/9789264203709-en

OECD (2018). Test No. 443: Extended One-Generation Reproductive Toxicity Study. *OECD Guidelines for the Testing of Chemicals, Section 4*. OECD Publishing, Paris. doi:10.1787/9789264185371-en

Ogungbemi, A. O., Teixido, E., Massei, R. et al. (2020). OptimiƟzation of the spontaneous tail coiling test for fast assessment of

neurotoxic effects in the zebrafish embryo using an automated workflow in KNIME®. *Neurotoxicol Teratol 81*, 106918. doi:10.1016/j.ntt.2020.106918

Ohno, S., Ikenaka, Y., Onaru, K. et al. (2020). Quantitative elucidation of maternal-to-fetal transfer of neonicotinoid pesticide clothianidin and its metabolites in mice. *Toxicol Lett 322*, 32-38. doi:10.1016/j.toxlet.2020.01.003

Osterauer, R. and Köhler, H. R. H.-R. (2008). Temperature-dependent effects of the pesticides thiacloprid and diazinon on the embryonic development of zebrafish (Danio rerio). *Aquat Toxicol 86*, 485-494. doi:10.1016/j.aquatox.2007.12.013

Özdemir, H. H., Kara, M., Yumrutas, O. et al. (2014). Determination of the effects on learning and memory performance and related gene expressions of clothianidin in rat models. *Cogn Neurodyn 8*, 411-416. doi:10.1007/s11571-014-9293-1

Paine, M. (2017). Therapeutic disasters that hastened safety testing of new drugs. *Clin Pharmacol Ther 101*, 430-434. doi:10.1002/cpt.613

Palpant, N. J., Hofsteen, P., Pabon, L. et al. (2015). Cardiac development in zebrafish and human embryonic stem cells is inhibited by exposure to tobacco cigarettes and e-cigarettes. *PLoS One 10*, e0126259. doi:10.1371/journal.pone.0126259

Paparella, M., Bennekou, S. H. and Bal-Price, A. (2020). An analysis of the limitations and uncertainties of in vivo developmental neurotoxicity testing and assessment to identify the potential for alternative approaches. *Reprod Toxicol 96*, 327-336. doi:10.1016/j.reprotox.2020.08.002

Papke, R. L., Ono, F., Stokes, C. et al. (2012). The nicotinic acetylcholine receptors of zebrafish and an evaluation of pharmacological tools used for their study. *Biochem Pharmacol 84*, 352-365. doi:10.1016/j.bcp.2012.04.022

Parker, B. and Connaughton, V. P. (2007). Effects of nicotine on growth and development in larval zebrafish. *Zebrafish 4*, 59-68. doi:10.1089/zeb.2006.9994

Pisa, L., Goulson, D., Yang, E.-C. et al. (2021). An update of the worldwide integrated assessment (WIA) on systemic insecticides. Part 2: Impacts on organisms and ecosystems. *Environ Sci Pollut Res 28*, 11749-11797. doi:10.1007/s11356-017-0341-3

Raftery, T. D. and Volz, D. C. (2015). Abamectin induces rapid and reversible hypoactivity within early zebrafish embryos. *Neurotoxicol Teratol 49*, 10-18. doi:10.1016/j.ntt.2015.02.006

Richendrfer, H., Creton, R. and Colwill, R. M. (2014). The embryonic zebrafish as a model system to study effects of environmental toxicants on behavior. In C. Lessman and E. A. Carver (eds.), *Zebrafish* (245-265). Nova Science Publishers, Inc. https://www.novapublishers.com/wp-content/uploads/2019/05/978-1-63117-558-9_ch12.pdf

Rihel, J., Prober, D. A., Arvanites, A. et al. (2011). Zebrafish behavioural profiling links drugs to biological targets and rest/wake regulation. *Nat Chem Biol 327*, 348-351. doi:10.1126/science.1183090.Zebrafish

Russell, W. M. S. and Burch, R. L. (1959). *The Principles of Humane Experimental Techniques*. London, UK: Methuen.

Saint-Amant, L. and Drapeau, P. (1998). Time course of the development of motor behaviors in the zebrafish embryo. *J Neurobiol 37*, 622-632. doi:10.1002/(SICI)1097-4695(199812)37:4<622::AID-NEU10>3.0.CO;2-S

Sano, K., Isobe, T., Yang, J. et al. (2016). In utero and lactational exposure to acetamiprid induces abnormalities in socio-sexual and anxiety-related behaviors of male mice. *Front Neurosci 10*, 228-240. doi:10.3389/fnins.2016.00228

Selderslaghs, I. W. T., Hooyberghs, J., De Coen, W. et al. (2010). Locomotor activity in zebrafish embryos: A new method to assess developmental neurotoxicity. *Neurotoxicol Teratol 32*, 460-471. doi:10.1016/j.ntt.2010.03.002

Selderslaghs, I. W. T., Hooyberghs, J., Blust, R. et al. (2013). Assessment of the developmental neurotoxicity of compounds by measuring locomotor activity in zebrafish embryos and larvae. *Neurotoxicol Teratol 37*, 44-56. doi:10.1016/j.ntt.2013.01.003

Sheets, L. P. (1994). An acute oral neurotoxicity screening study with technical grade imidacloprid (NTN 33893) in rats. Unpublished report from Miles Inc. report No. MOB7221, dated 16 February 1994, GLP, supplement report No. MOB7221, dated 7 June 1994. Submitted to WHO by Bayer AG, Mannheim, Germany.

Sobanska, M., Scholz, S., Nyman, A.-M. M. et al. (2018). Applicability of the fish embryo acute toxicity (FET) test (OECD 236) in the regulatory context of registration, evaluation, authorisation, and restriction of chemicals (REACH). *Environ Toxicol Chem 37*, 657-670. doi:10.1002/etc.4055

Sparks, T. C. and Nauen, R. (2015). IRAC: Mode of action classification and insecticide resistance management. *Pestic Biochem Physiol 121*, 122-128. doi:10.1016/j.pestbp.2014.11.014

Strähle, U., Scholz, S., Geisler, R. et al. (2012). Zebrafish embryos as an alternative to animal experiments – A commentary on the definition of the onset of protected life stages in animal welfare regulations. *Reprod Toxicol 33*, 128-132. doi:10.1016/j.reprotox.2011.06.121

Svoboda, K. R., Vijayaraghavan, S. and Tanguay, R. L. (2002). Nicotinic receptors mediate changes in spinal motoneuron development and axonal pathfinding in embryonic zebrafish exposed to nicotine. *J Neurosci 22*, 10731-10741. http://www.ncbi.nlm.nih.gov/pubmed/12486166

Takada, T., Yoneda, N., Hirano, T. et al. (2018). Verification of the causal relationship between subchronic exposures to dinotefuran and depression-related phenotype in juvenile mice. *J Vet Med Sci 80*, 720-724. doi:10.1292/jvms.18-0022

Tanaka, T. (2012). Reproductive and neurobehavioral effects of clothianidin administered to mice in the diet. *Birth Defects Res Part B Dev Reprod Toxicol 95*, 151-159. doi:10.1002/bdrb.20349

Terayama, H., Endo, H., Tsukamoto, H. et al. (2016). Acetamiprid accumulates in different amounts in murine brain regions. *Int J Environ Res Public Health 13*, 937. doi:10.3390/ijerph13100937

Tian, X., Hong, X., Yan, S. et al. (2020). Neonicotinoids caused oxidative stress and DNA damage in juvenile Chinese rare minnows (Gobiocypris rarus). *Ecotoxicol Environ Saf 197*, 110566. doi:10.1016/j.ecoenv.2020.110566

Tomizawa, M. and Casida, J. E. (2005). Neonocotinoid insecticide toxicology: Mechanisms of selective action. *Annu Rev Phar-*

macol Toxicol 45, 247-268. doi:10.1146/annurev.pharmtox.45.120403.095930

Vargesson, N. (2015). Thalidomide-induced teratogenesis: History and mechanisms. *Birth Defects Res Part C Embryo Today Rev 105*, 140-156. doi:10.1002/bdrc.21096

Vargesson, N. (2019). The teratogenic effects of thalidomide on limbs. *J Hand Surg Eurl 44*, 88-95. doi:10.1177/1753193418805249

Vignet, C., Cappello, T., Fu, Q. et al. (2019). Imidacloprid induces adverse effects on fish early life stages that are more severe in Japanese medaka (Oryzias latipes) than in zebrafish (Danio rerio). *Chemosphere 225*, 470-478. doi:10.1016/j.chemosphere.2019.03.002

Vliet, S. M., Ho, T. C. and Volz, D. C. (2017). Behavioral screening of the LOPAC1280 library in zebrafish embryos. *Toxicol Appl Pharmacol 329*, 241-248. doi:10.1016/j.taap.2017.06.011

von Hellfeld, R., Brotzmann, K., Baumann, L. et al. (2020). Adverse effects in the fish embryo acute toxicity (FET) test: A catalogue of unspecific morphological changes versus more specific effects in zebrafish (Danio rerio) embryos. *Environ Sci Eur 32*, 122. doi:10.1186/s12302-020-00398-3

Vorhees, C. V., Williams, M. T., Hawkey, A. B. et al. (2021). Translating neurobehavioral toxicity across species from zebrafish to rats to humans: Implications for risk assessment. *Front Toxicol 3*, 629229. doi:10.3389/ftox.2021.629229

Wang, Y., Yang, G., Dai, D. et al. (2017). Individual and mixture effects of five agricultural pesticides on zebrafish (Danio rerio) larvae. *Environ Sci Pollut Res 24*, 4528-4536. doi:10.1007/s11356-016-8205-9

Wang, Y., Li, X., Yang, G. et al. (2020). Changes of enzyme activity and gene expression in embryonic zebrafish co-exposed to beta-cypermethrin and thiacloprid. *Environ Pollut 256*, 113437. doi:10.1016/j.envpol.2019.113437

Welsh, L., Tanguay, R. L. and Svoboda, K. R. (2009). Uncoupling nicotine mediated motoneuron axonal pathfinding errors and muscle degeneration in zebrafish. *Toxicol Appl Pharmacol 237*, 29-40. doi:10.1016/j.taap.2008.06.025

Wilt, F. H. and Hake, S. (2003). *Principles of Developmental Biology*. W.W. Norton & Company.

Wu, S., Li, X., Liu, X. et al. (2018). Joint toxic effects of triazophos and imidacloprid on zebrafish (Danio rerio). *Environ Pollut 235*, 470-481. doi:10.1016/j.envpol.2017.12.120

Yoneda, N., Takada, T., Hirano, T. et al. (2018). Peripubertal exposure to the neonicotinoid pesticide dinotefuran affects dopaminergic neurons and causes hyperactivity in male mice. *J Vet Med Sci 80*, 634-637. doi:10.1292/jvms.18-0014

Yoo, M. H., Rah, Y. C., Park, S. et al. (2018). Impact of nicotine exposure on hair cell toxicity and embryotoxicity during zebrafish development. *Clin Exp Otorhinolaryngol 11*, 109-117. doi:10.21053/ceo.2017.00857

Yoshida, H. (1989). Hydrolysis of NTN 33893. Report No. NR1276. Japan.

Zhang, H. and Zhao, L. (2017). Influence of sublethal doses of acetamiprid and halosulfuron-methyl on metabolites of zebrafish (Brachydanio rerio). *Aquat Toxicol 191*, 85-94. doi:10.1016/j.aquatox.2017.08.002

Zindler, F., Beedgen, F., Brandt, D. et al. (2019a). Analysis of tail coiling activity of zebrafish (Danio rerio) embryos allows for the differentiation of neurotoxicants with different modes of action. *Ecotoxicol Environ Saf 186*, 109754. doi:10.1016/j.ecoenv.2019.109754

Zindler, F., Beedgen, F. and Braunbeck, T. (2019b). Time-course of coiling activity in zebrafish (Danio rerio) embryos exposed to ethanol as an endpoint for developmental neurotoxicity (DNT) – Hidden potential and underestimated challenges. *Chemosphere 235*, 12-20. doi:10.1016/j.chemosphere.2019.06.154

Zindler, F., Stoll, S., Baumann, L. et al. (2020). Do environmentally relevant concentrations of fluoxetine and citalopram impair stress-related behavior in zebrafish (Danio rerio) embryos? *Chemosphere 261*, 127753. doi:10.1016/j.chemosphere.2020.127753

Data availability

Original datasets of the current study and analyses generated are available in the BioStudies repository (https://wwwdev.ebi.ac.uk/biostudies/EU-ToxRisk/).

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the grant agreement No. 681102. The paper reflects only the authors' views, and the European Commission is not responsible for any use that may be made of the information it contains. For the publication fee we acknowledge the financial support provided by the Deutsche Forschungsgemeinschaft within the funding programme "Open Access Publikationskosten" as well as by Heidelberg University.

Author contributions

RvH conceptualized the experiments, created the figures and tables, and drafted the manuscript. VO conducted the experiments, analyzed the data, created some of the figures and tables, and conducted literature research. SB and MAL conducted the medium sample analysis at Cyprotex, with the involvement of CB and PW. AF, SHB and TB significantly contributed to the manuscript and data visualization and manuscript review. TB further provided funding and laboratory facilities. All authors read and approved the final manuscript..