von Hellfeld et al.: Zebrafish Embryo Neonicotinoid Developmental Neurotoxicity in the FET Test and Behavioral Assays

Supplementary Data

Video S1: Video example of the coiling assay set-up at 3x normal speed. Video taken after 38 h nicotine exposure: doi:10.14573/altex.2111021s2

Video S2: Video example of the swimming assay set-up at 3x normal speed. Video taken after 110 h nicotine exposure doi:10.14573/altex.2111021s2

Fig. S1: Overview of the neurogenesis of zebrafish embryos, as the basis of behavioral assays such as the coiling and the swimming assay
Figure created with biorender.com
Fig. S2: Representation of lordosis (L), kyphosis (K), scoliosis (S) pericardial edema (PE) and craniofacial deformation (CD) in the FET test observed in zebrafish (*Danio rerio*) embryos between 72 and 120 hpf.

Scale bar: 100 µm.

|                | Negative control | 50 µM Nicotine | 100 µM Nicotine |
|----------------|------------------|----------------|-----------------|
| 72 hpf         | K                | K              |                 |
| 96 hpf         | L, S, PE         | L, S, PE       |                 |
| 120 hpf        | L, PE, CD        | L, PE          |                 |

K, kyphosis; L, lordosis; S, scoliosis; PE, Pericardial edema; CD, craniofacial deformation.
Tab. S1: Overview of the chemical properties of the test compounds

| Compound      | g/Mol<sup>a</sup> | CAS no.       | Chemical class | Log K<sub>ow</sub><sup>b</sup> | Water solubility<sup>b</sup> | Stability and biodegradability<sup>c</sup> |
|---------------|------------------|---------------|----------------|-------------------------------|-------------------------------|-----------------------------------------------|
| Acetamiprid   | 222.68           | 160430-64-8   | Chloropyridyl  | 0.8                           | 3 g/L                         | Photolysis half-life time: 34 d at 25°C, pH 7 |
| Clothianidin  | 249.68           | 637-07-0      | Chlorothiazole | 0.7                           | 300 mg/L                      | Photolysis half-life time: < 1 d |
| Dinotefuran   | 202.21           | 165252-70-0   | Furanyl        | n/a                           | 39.8 g/L                      | n/a                                           |
| Imidacloprid  | 255.66           | 138261-41-3   | Imidazolidine  | 0.57                          | 600 mg/L                      | Almost entirely in cation form at pH 7-9; hydrolytically stable at pH 5-11 |
| Nicotine      | 163.23           | 54-11-5       | Dinitrogen alkaloid | 1.17                      | 1 g/mL                        | Lacks functional groups for hydrolysis; volatilization not expected |
| Thiacyclidine | 252.72           | 111988-49-9   | Chloropyridyl  | 1.26                          | 185 mg/L                      | Half-life time: 10-63 d |
| Thiamethoxam  | 291.71           | 153719-22-4   | Chlorothiazole | -0.13                         | 4.1 g/L                       | Hydrolytically stable at pH 5 with a half-life time: 200-300 d; at pH 9: half-life time a few days |

n/a, No information found for this character of the compound; <sup>a</sup> Sigma Aldrich: https://www.sigmaaldrich.com; <sup>b</sup> PubChem: https://pubchem.ncbi.nlm.nih.gov; <sup>c</sup> US National Library of Medicine (ToxNet): https://www.nlm.nih.gov/toxnet

Tab. S2: Details of analytical determination of exposure concentrations in the zebrafish (Danio rerio) exposure experiments, the mobile phases, and gradient profile

| Compound      | Accurate mass | Parent > daughter transition | Cone voltage (V) | Collision energy (eV) | Ion mode | UHPLC gradient or GC | Limit of detection (µM) |
|---------------|---------------|------------------------------|------------------|-----------------------|----------|----------------------|-------------------------|
| Acetamiprid   | 222.67        | 224.04>126.93               | 21               | 20                    | ESP+     | Formic_FAST_B2       | 0.75                    |
| Clothianidin  | 249.68        | 250.91>169.97               | 14               | 10                    | ESP+     | Formic_FAST_B2       | 0.5                    |
| Dinotefuran   | 202.21        | 205.05>129.03               | 14               | 10                    | ESP+     | Formic_FAST_B2       | 0.5                    |
| Imidacloprid  | 255.66        | 256.99>175.97               | 14               | 20                    | ESP+     | Formic_FAST_B2       | 0.5                    |
| Nicotine      | 162.23        | 163.06>129.99               | 42               | 20                    | ESP+     | Acetate_FAST_B2      | 0.3                    |
| Thiacyclidine | 252.72        | 254.00>126.9                | 21               | 30                    | ESP+     | Formic_FAST_B2       | 0.75                   |

Mobile phase A: 10 mM ammonium formate + 0.1% v/v formic acid in water
Mobile phase B: methanol

Gradient profile:

| Time (min) | Flow rate (µL/min) | % Mobile phase A | % Mobile phase B | Gradient profile |
|------------|-------------------|------------------|------------------|-----------------|
| 0.00       | 1000              | 100              | 0                | 6               |
| 0.03       | 1000              | 100              | 0                | 6               |
| 0.60       | 1000              | 5                | 95               | 5               |
| 0.65       | 1000              | 5                | 95               | 6               |
| 0.80       | 1000              | 100              | 0                | 11              |
| 0.90       | 1000              | 100              | 0                | 1               |
| Setting          | Parameter            |
|------------------|----------------------|
| **Video settings** |                      |
| Basler acA1920-155um | 1600x1200            |
| Gain auto        | Off                  |
| Gain selector    | All                  |
| Gain             | 1.00000              |
| Black level selector | All            |
| Black level      | 0.00000              |
| Gamma            | 1.00000              |
| Digital shift    | 4                    |
| **Detection settings** |                |
| Activity onset   | 2%                   |
| Activity offset  | 0.5%                 |
| Minimum inter peak interval | 100 ms         |
| Minimum peak duration | 0 ms               |
| **Swimming assay** |                      |
| Basler acA1300-60 gm | 1280 x 960          |
| Gain auto        | Off                  |
| Gain selector    | Analog All           |
| Gain (raw)       | 0                    |
| Black level selector | All            |
| Black level (raw) | 50                  |
| Gamma enable     | Disabled             |
| Gamma selector   | User                 |
| Gamma            | 1                    |
| Digital shift    | 1                    |
| **Detection Settings** |                   |
| Method           | DanioVision          |
| Detection sensitivity | 180               |
| Activity threshold | 100                |
| Activity background noise filter | 5               |
| Compression artifacts filter | On              |
Tab. S4: Summary of all studies referred to in the discussion
Methodological information, endpoints assessed as well as significant findings. Concentrations not converted into molarity for the present study.

| Model organism | Methodology | Endpoint(s) assessed | Significant finding(s) | Reference |
|----------------|-------------|----------------------|-------------------------|-----------|
| Wildtype (AB) zebrafish | Reared at 28°C, with 14/10 h light/dark cycle. Eggs collected and reared in embryo medium before use. Clutch 1: embryos at 6 hpf exposed to acetamiprid in 200 µL solution in 96-well plate until 12 hpf. Clutches 2-5: 20 embryos per replicate raised in 6-well plates with 5 mL solution (54, 107, 263, 443, 537, 760, and 974 mg/L) for 12 h without medium renewal. Heart rate measured at 48, 60 and 72 hpf in 10-second videos. Body length measured after 120 h, coiling examined at 17 and 27 h post fertilization (hpf), touch response examined at 27, 36, and 48 hpf (after dechorionation). | Morphology: mortality, lethal concentration (LC), malformations, hatching, heart rate, and body length Behavior: spontaneous tail coiling and touch response | - Morphology: 374 mg/L induced significant mortality at 120 hpf 760 mg/L induced complete mortality. Hatching only affected > 547 mg/L 120 hpf LC50: 518 mg/L 120 hpf EC50: 323 mg/L Effects: bent spine, uninflated swim bladder, pericardial and yolk sac edema > 107 mg/L reduced heart rate at 48, 60 and 72 hpf Body length decreased in dose-dependent manner from 54 mg/L Behavior: delayed onset of spontaneous movement, inhibiting response at >760 mg/L Recovery < 760 mg/L. No movement at 974 mg/L • Absolute brain weight of newborn ♂ pups significantly lower after acetamiprid treatment • Cortical plate thickness significantly reduced in pups of maternal mice treated from GD 6 to 13 • Significant decrease in cell cycle exit at 5 mg/kg, linking cortical plate hypoplasia to decreased neurogenesis • Prenatal exposure altered neuronal distribution, but not number of neurons on PND 14 • On PND 14, pups showed increased number of amoeboid-type microglia, without showing changes in numbers of ramified or transition-type microglia and total microglia | Ma et al., 2019 |
| ICR mice | 10-wk old mice housed in 24°C, 55% humidity and a 12/12 light/dark cycle. Acetamiprid administered via oral gavage (5 mL/kg body weight) for varying times between gestational days (GD) 3 and 18. Pregnant mice sacrificed and embryos of postnatal day (PND) 14 examined. | Histology: fetal tissue & weight Immunohistochemistry: β-tubulin, anti-Ki67, bromodeoxyuridine, anti-bromodeoxyuridine, anti-Iba1, antiCD11b, and anti-CD206 | • Number of sexual behaviors of ♂ ♂ significantly increased in low-dose group (especially mean mount numbers) • Aggression level in low-dose group ♂ ≥ significantly increased in total duration and number of bouts compared to high-dose | Kagawa and Nagao, 2018 |
| C57BL/6J mice | Mice housed at 24°C, 50% humidity with a 12/12 h light/dark cycle. 0, 1, 10 mg/kg acetamiprid administered by oral gavage in water from GD 6 to PND 21. Pups weaned 2-3 h after last dosing (d 21). ♂ sex behavior towards hormone-treated ♀ tested 12-14 d after final dosing for 3 wks (weekly 30-minute trials), 5-7 d after ♂ sex behavior test, aggressive | Morphology: Body weight (at birth, at meaning and at 23-26 weeks of age), brain weight (at 21 d of age) Behavior: ♂ sexual behavior, ♂ aggressive behavior, ♀ sex behavior, LDT | • Number of sexual behaviors of ♂ ♀ ♀ significantly increased in low-dose group (especially mean mount numbers) | Sano et al., 2016 |
| Model organism | Methodology | Endpoint(s) assessed | Significant finding(s) | Reference |
|----------------|-------------|---------------------|------------------------|------------|
| behavior towards ♂ intruder mice tested weekly for 3 wks in 9 trials of 15 min. 12-14 wks ♀♀ ovariectomized and 2 wks later tested for sexual behavior towards experienced ICR/JCL ♂ mice (3 trials). Light-dark test (LDT) examined emotional behavior in enclosed dark and open-top light compartments. | test, and behavioral flexibility | and control group • Low- and high-dose groups spent significantly more time in light compartment of LDT test than controls • ♂ mice of both dosing groups traveled longer distances in light compartment than the control group | | |
| Mosiplan® (technical product of acetamiprid) | 3-wk old mice were housed at 22-24°C, in 50-60% humidity, on a 12 h light/dark cycle. Dosed via drinking water with 0.594 mg/mL (0.66 mL Mosiplan SP/200 mL water) or 5.94 mg/mL (6.66 mL Mosiplan SP/200 mL water) for 180 days. | Morphology; body weight, testis weight (histological and biochemical analysis) Serum samples from heart RT-PCR: of testis and pituitary gland. Examined Ki67, Top2a, Lhr, Star, Cyp11a1, Cyp17a1, and Hsd17b1 | • 5.94 mg/mL significantly reduced body weight • Abnormal seminiferous epithelium was observed in some seminiferous tubules after treatment • Cell proliferation marker Ki67 reduced in higher exposure group, Top2a affected by both doses • Testosterone metabolism affected: higher dose of acetamiprid downregulated Lhr, Star, Cyp11a1, and Hsd17b1 | Terayama et al., 2016 |
| Acetamiprid, clothianidin, dinotefuran and thiamethoxam | Reared at 28°C with a 12/12 h light/dark cycle. Exposure for 24 h with 7-d old larvae, in 48-well plate with 1 larva per 1 mL well. Vibrational startle response assay (VSRA) conducted at 8 dpf (tapping intensity at 8 and 50 vibrational sequences). Exposure concentrations: acetamiprid (40, 400 µg/L), clothianidin (3, 30 µg/L), dinotefuran (0.13, 1.3 µg/L), thiamethoxam (0.19 1.9 µg/L); environmentally relevant (ERC) and ‘worst case scenario’ (WSC, 10-fold of ERC) | Behavior: Habituation and startle response to stimuli | • Acetamiprid: At ERC habitation significantly reduced. At WSC, startle response increased, habitation reduced • Clothianidin: At WSC, startle response increased • Dinotefuran: At ERC and WSC, habitation reduced | Faria et al., 2020 |
| Wildtype zebrafish | In vitro mice: Adult 5-6 wks ♀ mice were mated and sacrificed to isolate the embryos at 2-cell stage. In vitro rabbits: Adult ♂ rabbits were mated; embryos flushed from oviducts 20 h post-coitum; embryos exposed to 0, 0.1, 1, 10, 100 µM for 72 h. | Development | Mouse: • 100 µM affected embryonic development in vitro, reducing number of embryos reaching blastocyst stage: thiacloprid > clothianidin > acetamiprid > thiamethoxam • 10 µM thiamethoxam also affected development • In vivo exposure decreased the cell number in blastocysts at both concentrations Rabbit: • 100 µM thiacloprid in vitro decreased the cell numbers in blastocysts | Babefová et al., 2017 |
| ICR (CD-1 IGS) mice and New Zealand White rabbits | In vitro mice: Adult 5-6 wks ♀ mice were mated and sacrificed to isolate the embryos at 2-cell stage. In vitro rabbits: Adult ♀ rabbits were mated; embryos flushed from oviducts 20 h post-coitum; embryos exposed to 0, 0.1, 1, 10, 100 µM for 72 h. | Development | Mouse: • 100 µM affected embryonic development in vitro, reducing number of embryos reaching blastocyst stage: thiacloprid > clothianidin > acetamiprid > thiamethoxam • 10 µM thiamethoxam also affected development • In vivo exposure decreased the cell number in blastocysts at both concentrations Rabbit: • 100 µM thiacloprid in vitro decreased the cell numbers in blastocysts | Babefová et al., 2017 |
| clothianidin | Housed at 23°C at 50% humidity on a 14/10 h light/dark cycle. ♀ mice on GD 1 administered 65 mg/kg/day clothianidin via oral gavage either in a single-dose administration or daily dosing for 4 or 9 d. Single-dose group | Blood analysis | • Clothianidin and 5 metabolites found in dam and fetus blood samples • Concentrations of clothianidin higher in animals sacrificed 1 h after administration | Ohno et al., 2020 |
Model organism  | Methodology                                                                 | Endpoint(s) assessed                                      | Significant finding(s)                                                                 | Reference          
--- | --- | --- | --- | --- 
Crlj: CD1 mice | 4-wk mice housed at 25°C, 50% humidity with a 12 h light/dark cycle. Clothianidin was administered via diet (0.003, 0.006, and 0.012%). F0 generation was examined on d 0, 2, 4, 7, 21, 28, and 30 during preconception. At 9-weeks, mating conducted, dams weighed weekly during gestation and lactation. F1 examined 0, 4, 7, 14, and 21 PNDs, as well as 4- and 11-weeks post-weaning. Weaning at 4 weeks of age; one ♀ and one ♂ randomly selected for continued treatment of each litter. | Morphology: size, mortality, weight, sex ratio  
Behavior: Surface righting, negative geotaxis, cliff avoidance, swimming behavior, olfactory orientation | • F0 ♀ increased exploratory behavior, average time of movement, number of rearing, and rearing time at 8 wks in concentration-dependent manner  
• F1 ♀ average body weight increased in low-dose group at postnatal day 7; mid-dose group ♀ body weight increased significantly at PND 4 and 7  
• F1 ♂ body weight increased in low and mid-dose groups at PND 4 and 7  
• Development of swimming head angles delayed in mid-dose offspring at PND 7, and time taken for olfactory orientation at PND14 accelerated in mid-dose offspring  
• ♂ offspring surface righting at PND 4 in the low-dose group; swimming head angle development in low- and mid-dose groups at PND 7 accelerated; negative geotaxis affected; olfactory orientation delayed in mid-dose group; number of rearing at 3 wks increased  
• ♀ exploratory behavior at 8 weeks increased  
• At 10 wks, ♂♂ horizontal inactivity in the low-dose group; ♀♀ less active in average speed and rearing time for mid-dosed group | Tanaka, 2012 
C57BL/6N mice | ♀ mice housed in 23°C and a 12 h light/dark cycle; clothianidin orally administered at wks 9-10 (0, 5, 50 mg/kg body weight). Elevated plus-maze test conducted 1 h after administration, 2 h later. | Behavior: Elevated plus-maze test, and vocalization  
Neuroactivity | • 5 mg/kg dosed mice affected total distance moved in the maze, whilst 50 mg/kg also reduced the number of entries into the open arms  
• 50 mg/kg mice spontaneously emitted vocalization in the maze when placed in open arms  
• Only the medial blade of the dentate gyrus and paraventricular thalamic nucleus showed increases in the c-fos immunoreactive nucleus per area in 50 mg/kg dosed mice | Hirano et al., 2018
| Model organism | Methodology | Endpoint(s) assessed | Significant finding(s) | Reference |
|----------------|-------------|----------------------|-------------------------|-----------|
| Albino Wistar rats | Newborn ♀ pups dosed with 2, 8, or 24 mg/kg body weight via gavage from PND 7 until 97. Additionally, 8-9 wks ♀ rats dosed with 2, 8, or 24 mg/kg body weight via gavage for 3 months. | Behavior: Morris maze, probe trials  
Gene expression: hippocampus expression of grm1, m1, syp, and gap-43 | • Escape latency of adult mice on d 1 and 3 affected  
• Infant mice spent less time in the target quadrant of the probe trial with increasing dosing | Özdemir et al., 2014 |
| C75BL/6NrSlc mice | - mice were dosed with 0, 100, 500, or 2500 mg/kg/day from 3 to 8 weeks of age via drinking water. Behavioral assays were conducted after the 6 weeks of exposure. Sacrifices were performed the following day. | Behavior: Open field test and Y-maze test  
Brain samples: weight, immunoreactivity to tyrosine hydroxylase (TH) in substantia nigra, and dopamine (DA) receptor D1 and D2 in striatum | • TH immunoreactivity enhanced in the exposure groups | Yoneda et al., 2018 |
| Chinese rare minnow | Reared at 25°C on a 16/8 h light/dark cycle. 2 mo fish exposed to 0.1, 0.5 or 2.0 mg/L for 60 d, fed daily. Fish sacrificed and livers collected for oxidative stress assessment and qRT-PCR. | Oxidative stress: liver glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT)  
qRT-PCR: CUT2Zn-sod, Mn-Sod, cat, gpx1, gcl, cyp1a, gstm, gly1a, and β-actin  
Genotoxicity: comet assay | Dinotefuran:  
• 0.1 mg/L reduced Cu/zn-sod and gstm expression, 0.5 mg/L reduced cat, cyp1a, and gstm1. 2 mg/L reduced Mn-sod expression  
• SOD and GSH activity increased  
• CAT and MDA activity reduced  
Imidacloprid:  
• 0.5 mg/L reduced gstm expression  
• 2 mg/L reduced Cu/Zn-sod, gpx-1, cyp1a, and gstm expression  
• SOD and GSH activity increased  
• MDA activity decreased | Tian et al., 2020 |
| Wildtype (AB) zebrafish | Reared at 26°C with a 14/10 h light/dark cycle. 96 h FET test (OECD TG 236) with 24 h medium renewal. | Morphology: Mortality, LC, developmental alterations.  
Biochemical and molecular tests: SOD, CAT, glutathione-S-transferase (GST), carboxylesterase (CarE), cytochrome p450 (Cyp450), Caspase 3, Caspase 9, vitellogenin (VTG), triiodothyronine (T3), and thyroxine (T4)  
Quantitative (q) PCR: Caspase3, Caspase9, Mn-sod, Cu/Zn-sod, cat, gpx, bcl-2, ucp-2, cas3, cas9, bax, Apaf.1, p53, CYCL-CIC, CC-chem, IL-1ß, IL-8, TRα, Embryonic LC50:121.6 mg/L  
Larval LC50:128.9 mg/L  
Juvenile LC50:1.13 26.39 mg/L  
Adult LC50:76.08 mg/L  
Reduced CarE and CAT activity, increased Cyp450, Caspase3 and Caspase9 activity.  
Decreased relative Mn-sod, Gpx, cas3, cas9, CXCL-CIC, CC-chem, IL-1ß, IL-8, Dio1, Dio2, and Isth mRNA levels, increased Cu/Zn-sod, bcl-2, ucp-2, bax, p53, and TRβ mRNA levels | Wu et al., 2018 |
| Model organism                  | Methodology                                                                 | Endpoint(s) assessed                                                                                                                                                                                                                                                                                                                                 | Significant finding(s)                                                                                                                                                                                                                      | Reference                        |
|--------------------------------|-------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------|
| Wildtype zebrafish and Japanese medaka | Imidacloprid tested at 0.2-200 µg/L  
Zebrafish: Reared at 28°C, 14/10 h light/dark cycle. Eggs were collected and exposure ended at 5 d post-fertilization (dpf). Swimming behavior was assessed at 5 dpf.  
Medaka: Reared at 26°C, 14:10 h light/dark cycle and exposure started at 13 hpf, ending at 14 dpf; swimming behavior assessed at 14 dpf. | Morphology: Survival and development, histology  
Behavior: Swimming behavior  
Biotransformation:  
Chorion assumed barrier to exposure for both species  
At the end of the exposures, about 15% of imidacloprid metabolized  
Zebrafish: Marked thickening of muscle fibers in 2000 µg/L treatment group  
Medaka: Transiently affected hatching at 7 and 8 dpf  
Lordosis/scoliosis, hemorrhaging, concentration-dependent jaw/skull deformation > 0.2 µg/L; bone and yolk edema, tail deformities > 20 µg/L  
Disorganization of retinal pigment epithelium > 0.2 µg/L  
Altered myomere structure, total body length affected > 0.2 µg/L  
Disorganization of retinal pigment epithelium > 0.2 µg/L  
Altered myomere structure, total body length affected > 0.2 µg/L | Vignet et al., 2019 |
| Leghorn chicken              | Method 1: Chicks at Hamburger-Hamilton (HH) stage 0 incubated with 500 µM imidacloprid at 38°C and 70% humidity.  
Method 2: 500 µM imidacloprid applied to one side of gastrula-stage embryos.  
Method 3: HH4 embryos exposed to 500 µM imidacloprid through injection into windowed egg in vivo and incubated for another 4.5 or 14 days.  
In situ hybridization, immunofluorescent staining, RT-PCR and western blots were performed. | Morphology: Mortality, growth, weight, and somite development  
Heart development: morphology, and cardiomyocyte differentiation  
Biochemistry: in situ for vhlmc, fata5, bmp2, and nkx2.5. Immunofluorescent staining with MF20, E-cadherin, and Laminin antibodies. RT-PCR for gata4, tbx5, vergfr2, bmp3 | Mortality increased to 50% by 14 d incubation; growth increased with treatment, but weight and somite development were reduced  
Ventricular wall and trabecular muscle thickness reduced  
On day 14, heart size and weight as well as whole embryo weight reduced  
Right ventricular wall thicker; no effect on left ventricular wall or interventricular septum  
Atypical C-looping in HH10 chicks  
Gata5 and nkx2.5 expression downregulated in imidacloprid-treated embryos (method 2)  
RT-PCR increased Wnt3a, and reduced gata4, tbx5, vergfr2, and bmp4 expression  
Western blot: inhibition of GATA4, GATA6, and TBX5  
Expression of E-cadherin extended to epiblast, mesoderm, and hypoblast  
RT-PCR: reduced N-cadherin and increased E-Cadherin expression  
Migration of cardiac progenitor cells inhibited; migration, polarization, and protrusion formation of cardiac cells suppressed in vitro | Gao et al., 2016 |
| Model organism | Methodology | Endpoint(s) assessed | Significant finding(s) | Reference |
|----------------|-------------|----------------------|------------------------|-----------|
| CD-1 mice      | 7-10 wks mice housed at 22°C on a 12/12 h light/dark cycle. On GD 3-6 osmotic mini-pumps implanted, dispensing 0.5 mg/kg/day imidacloprid. Offspring housed with respective ♀ until weaning on PND 21. Pup’s sex-matched and housed undisturbed until PND 42. On PND 43-47, open field test conducted. On PND 47-54, elevated plus maze conducted. On PND 61-67, forced swim test was conducted. Tube test conducted on PND 54-64. On PND 66-72, resident intruder test conducted. | Behavior: Open field test, elevated plus maze, forced swim test, tube test, resident intruder test Biochemistry: Serum and tissue concentrations | • Number of pups lower than in controls ♂ markedly lighter • Triglyceride serum concentrations reduced • Distance traveled in open field test increased • In forced swim test, imidacloprid reduced time spent immobile in both sexes • In the tube test, dosing significantly increased the winning percentage in both sexes • Resident intruder test: reduced attacks by residents count, duration, and fight time • Liver and brain concentrations in maternal mice and pups elevated, with maternal being higher | Burke et al., 2018 |
| Albino Wistar rats | Newborn and 9 wks ♂ rats treated with 0.5, 2, and 8 mg/kg body weight via gavage daily for 3 months. Buoyancy tested at PND 97 (newborns) or at 3 months of age, when sacrifice. | Learning: Morris maze, and probe trials | Infants: • 2 and 8 mg/kg increased latency in the Morris maze on d 3-5; 8 mg/kg affected probe trials Adults: • 8 mg/kg escape latencies longer on d 4 and 5 of the Morris maze; 8 mg/kg affected probe trials | Kara et al., 2015 |
| Sprague-Dawley [Sas:CD(SD)BD] rats | Single dosing of 0, 42, 150 or 310 mg/kg body weight via gavage. | Morphology: Mortality, development Serum analysis Behavior: Functional observational battery | • 310 mg/kg body weight: 14 rats died • Dose-related increase in incidence and severity of effects >150 mg/kg: tremor, nasal staining, uncoordinated gait, decreased activity, reactivity, urine staining, lower body temperature • Signs of toxicity observed on day 0 and resolved within 5 days • Dose-related decrease in motor and locomotor activity > 42 mg/kg for ♀ and >150 mg/kg for ♂ • 150 mg/kg: serum triglycerides decreased; survivors of highest dose decreased potassium and cholesterol concentrations (♀) and decreased alanine aminotransferase activity (♀, ♂) | Sheets, 1994 |
| Imidacloprid & nicotine | Wildtype (AB and 5D) zebrafish | Behavior: Larvae: swimming activity in response to environmental stimuli Adolescents: startle response and habituation, novel tank exploration, and | Larval activity: • > 45 µM nicotine and imidacloprid reduced activity during dark phase Adolescent neurobehavior: • > 45 µM imidacloprid and 45 µM nicotine | Crosby et al., 2015 |
| Model organism | Methodology                                                                 | Endpoint(s) assessed                                                                 | Significant finding(s)                                                                                                                                                                                                 | Reference |
|----------------|------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| Wildtype zebrafish | 96-well plate with un-dosed water for behavioral examination 24 h later. Embryos for adolescent and adult assessment reared in un-dosed water. | shoaling behavior Adults: startle response and habituation, novel tank exploration, shoaling behavior, and predator avoidance | exposure induced hyperactivity in the startle response assay Individuals treated with imidacloprid spent more time in min 4 and 5 of the novel tank assay near the tank floor 45 µM nicotine-exposed fish swam further than controls in shoaling assay Adult neurobehavior: > 45 µM nicotine and 60 µM imidacloprid induced fish to remain closer to tank floor in novel tank assay | Faria et al., 2019 |
| Wildtype zebrafish | Rearing in 28°C on a 12 h light/dark cycle. Embryos treated with 50 µM nicotine or 5.25 or 50 µM imidacloprid for 24 h from day 7 or 8. Experiments conducted in 48-well plates. Toxicity examined at 8 dpf. Startle response tested using high intensity tapping followed by vibrations of the plate. | Morphology: mortality, developmental changes, impaired swimming Behavior: startle response swimming (ranked compared to control) | Nicotine-exposed larvae moved more during the VSRA > 25 µM imidacloprid reduced distance moved in a concentration-dependent manner |           |
| Wildtype zebrafish | Rearing in 250 mL beakers under 0, 10, 20 or 40 µM nicotine exposure at 28°C, 14/10 h light/dark cycle (40-50 embryos per group). Daily renewal of 50-75% of the medium. Feeding with Paramecium from 72 hpf. Sacrificed at 10 dpf. | Morphology: notochord length, dry weight, hatching success, morphological alterations, pigmentation Behavior: startle response swimming (ranked compared to control) | Exposure reduced overall egg survival Notochord length, dry weight, and eye diameter reduced by > 20 µM Hatching delayed with increasing concentration 40 µM: short or bent body axis, altered pigmentation > 20 µM: reduced startle response from day 5 | Parker and Connaughton, 2007 |
| Wildtype (AB) zebrafish | Embryos reared in exposure medium at 28°C, 14/10 h light/dark cycle from 24 to 120 hpf. Behavioral analyses conducted at 5 dpf without prior medium renewal. | Morphology: Mortality, LC, development Behavior: visual motor response to light changes in the swimming assay | LC<sub>50</sub>: at 24 hpf 0.47 mMol/l, at 48 hpf 45 mMol/l 10-40 mg/L: monotonic suppression of distance moved during the light and dark phases basal swimming phase compared to controls | Ali et al., 2012 |
| Wildtype (AB) and transgenic (TG) (brn3c:egfp and cmlc2:egfp) zebrafish | Reared at 28.5°C under 14/10 h light/dark cycle, with ~ 50 embryos per 100 mm petri dish. Embryotoxicity of 0, 5, 10, 20 and 40 µM nicotine assessed by 72 hpf (10 embryos per well, 80 embryos per treatment). | Embryo toxicity (wildtype): hatching rate, mortality, bent spine and tail, stunted growth, malformed yolk sacs, and edema Embryo toxicity (transgenic): heart malformation, heart rate Hair cell toxicity at 120 hpf: neuromast analysis, hair cell apoptosis and mitochondrial damage within hair cell Intracellular reactive oxygen species (ROS) | 1% embryo toxicity in 5 µM, 44% in 40 µM nicotine 120 hpf, 40 µM almost all embryos dead nicotine treatment increased hair cell damage Heart-beat rate reduced in concentration dependent manner (no heart malformation) In brn3c:egfp: decreased average number of hair cells in neuromasts ROS induced by 40 µM Kinocilia of hair cells destroyed after 40 µM exposure, fewer stereocilia bundles after 5 µM treatment | Yoo et al., 2018 |
| Model organism | Methodology | Endpoint(s) assessed | Significant finding(s) | Reference |
|----------------|-------------|---------------------|------------------------|-----------|
| Homozygous wildtype and islet-1 TG zebrafish | Rearing in untreated water with daily medium renewal. At 19-21 hpf embryos dechorionated manually. At 22 hpf, pigmentation inhibited with 1-phenyl-2-thiourea (PTU) and 0-22 µM nicotine for exposure groups. Where nicotinic receptor antagonists (MLA and DHßE at 100 nM, 2 and 20 µM) were used: applied 2 h before nicotine exposure. | Ultrasound changes: scanning electron microscopy | • Reduced overall growth from 42 hpf onwards  
• 33 µM induced muscular response, but lack of swimming at 42 hpf, remained paralyzed until 120 hpf  
• Partial recovery when exposed between 22 and 66 hpf and allowed to recover by 120 and 168 hpf  
• > 66 hpf only few GFP-expressing motoneurons in spinal cord, with increased expression in rescued embryos at 120 hpf  
• 15 and 33 µM reduced% ventral myotomes with GFP-expressing axons  
• Continued expression of zn5 indicated delay in normal downregulation program  
• 33 µM reduced% of innervated dorsal segments at 66 hpf, with recovery potential | Svoboda et al., 2002 |
| Wildtype (TL, AB and WIK) and TG (isl1:gfp, fli1:gfp, and nbt:mapt-gfp) zebrafish | Reared at 28°C until 13 hpf, then at 25°C. Some embryos were dechorionated via enzymatic digestion exposed to 1-30 µM nicotine from 22 hpf, with daily renewal. For coiling response, control and dosed individuals were examined, as well as all groups after 3 min acute exposure to 5-30 µM, and after a recovery phase. Some embryos decapitated to determine tail movement alone. | Behavior: motor output (spinal musculature bend), and percentage of full movements (doublets)  
Immunochemistry: [3H]-nicotine uptake: measuring –(+) [N-methyl-3H] nicotine activity via liquid scintillation counting  
Influx and efflux: radioisotopes | • 33 µM exposure of non-dechorionated embryos induced paralysis by 66 hpf, with brief transient period of increased motor output  
• 5-30 µM produced increased muscle bends in dechorionated embryos at 27-28 hpf, but reduced percentage of doublets to almost zero, which recovered during washing  
• 30 µM caused 4-fold increase in musculature bend in dechorionated embryos at 22, 24, 25 and 26 hpf, but failed to induce the same response to 30 µM after a 2 h wash period, desensitizing the receptors  
• Tails from decapitated embryos exhibited increased musculature bends when placed in 5-30 µM nicotine  
• Exposure to high, followed by low concentration nicotine did not induce increased musculature bends after washing  
• Steady state of exposure for nicotine accumulation in embryos reached after 10 min, increasing with medium concentration, but always being less than | Thomas et al., 2009 |
| Model organism | Methodology | Endpoint(s) assessed | Significant finding(s) | Reference |
|----------------|-------------|----------------------|-------------------------|-----------|
| Wildtype (EkkWill) and TG (isl2b:gfp) zebrafish | Reared at 28°C with 14/10h light/dark cycle, exposed to 3-300 µM nicotine. For some assays, embryos reared in 0.002-0.0045% PTU for 24 h. Embryos placed in 100 mm petri dished for microinjection with morpholino antisense oligonucleotides (MOs). RT-PCR performed at 24 and 48 hpf. | Morphology: Mortality, development<br>Behavior: spinal musculature bends between 20 and 28 hpf, some dechorionated after nicotine exposure at 22 or 24 hpf; response to tactile stimuli of trunk at 31 hpf<br><br>In situ hybridization of PTU treated embryos for α 2A nAChR probes MOs targeted to the predicted translation site of nAChR α2 subunit, the splice blocking of the exon2-intron2 boundary, and a standard control)<br><br>Immunohistochemistry: zn8 (aka. zn5), zn1, zn1, F59, and zn12 | • α2A nAChR mRNA present in olfactory neurons and spinal cord from 19 hpf (probably Rohon beard neurons)<br>• Translated protein also found present in olfactory epithelium, spinal cord, and muscle<br>• Injection of α2A MO reduced α2A protein expression significantly in olfactory epithelium and Rohon beard neurons, successfully blocking expression of nAChR α2A subunit in vivo<br>• α2A morphants showed reduced bend rates immediately after exposure to 60 µM nicotine<br>• Between 20 and 22 hpf, nicotine-induced swim-like behavior was almost completely missing in α2A morphants, but by 23 hpf a significantly reduced motor response was elicited<br>• α2A MO did not disrupt formation of muscle-specific nAChRs<br>• Input elements (spinal neurons) produce nicotine-induced swim-like behavior, without affecting output elements (motoneurons and muscles) | Menelaou et al., 2014 |
| Homozygous wildtype (AB, WIK, and TL) and TG (isl1:gfp), and sofa potato (sop) zebrafish | Reared at 28°C on 14/10 h light/dark cycle. Untreated until 22 hpf, then exposed to 15 or 30 µM nicotine until 72 hpf. | Behavior: 48 hpf tail touch response<br><br>Live imaging of isl1 embryos<br><br>Morphology: via whole-mount immunohistochemistry (F59, F310, zn1, zn5, and anti-β2), and histology | • Exposed embryos had shortened dorsal/ventral axis with disorganized atrophic muscles<br>• Nicotine altered slow and fast muscles in wildtype and isl1 embryos<br>• In isl1 embryos pathfinding problems of secondary motoneuron axons after exposure to 15-30 µM nicotine until 72 hpf | Welsh et al., 2009 |
| Zebrafish and African frogs | Reared at 28°C. Zebrafish nAChR cDNA cloned for subunits α4, α2, β2, α7, α3 and β4. qPCR performed 1, 2, 3, 8 and 21 dpf. Mature Xenopus ovaries removed, and stage 5 oocytes isolated and injected with subunit cRNA, followed by up to 10 d of recovery. | Zebrafish nAChRs in Xenopus oocytes: Expression, electrophysiology, and functional responses<br><br>qPCR: β1a, β1b, and Elongation factor-1α (Elf1-α) | • All tested receptors responded well to > 3 µM acetylcholine<br>• Nicotine partial agonist for all heteromeric receptor subtypes, being most potent for α4β2 and least potent for muscle-type receptors<br>• Nicotine full agonist for α7 | Papke et al., 2012 |
| Wildtype (AB) zebrafish and RUES2 human Embryonic Stem Cells (ESC) | In vivo: Continuous exposure of embryos to tobacco smoke (TS), aerosol (AE) extracts (generated from cigarettes and e-cigarettes, containing 1 e-cigarette cartridge or 22 cigarettes) or nicotine until 72 hpf, with daily renewal. 14/10 h light/dark cycle, at 27.5°C. | In vivo: Morphology at 72 hpf: heart malformation, heart rate<br>Gene expression at 24 hpf | • 34 µM TS extract reduced survival at 24 hpf<br>• 34 µM TS and AE extracts reduced survival > 48 hpf | Palpant et al., 2015 |
| Model organism | Methodology | Endpoint(s) assessed | Significant finding(s) | Reference |
|----------------|-------------|---------------------|------------------------|-----------|
| *In vitro*: 1.7, 3.4, 6.8 and 13.7 µM nicotine from extracts from differentiation onset, and renewed daily. | *In vitro*: Gene expression, flow cytometry, immunofluorescence, cell stress assay | • 13.7 µM TS extract induced decreased hatching and pigmentation  
• TS and AE extracts induced heart defects, but only TS extracts reduced the heart rate  
• Only TS significantly affected gene expression (of *cmlc2, tnnt2, nkx2.5, mef2ca,* and *cx43*) | | |
| Virgin Sprague-Dawley rats | Experiment 1: Pregnant ♂♀ dosed with 0.05 mg/mL nicotine as source of drinking water. Dosed for the last 14, 6 or 4 d of pregnancy. Experiment 2, dosing continued post-delivery; both adult and fetal rats sacrificed at 21 or 22 PND. | Experiment 1: fetal body weight was measured, and brain and liver lipid and nitrogen determination on pooled organs  
Experiment 2: body weight | • 13.7 µM TS extract induced decreased hatching and pigmentation  
• TS and AE extracts induced heart defects, but only TS extracts reduced the heart rate  
• Only TS significantly affected gene expression (of *cmlc2, tnnt2, nkx2.5, mef2ca,* and *cx43*) | Mosier and Armstrong, 1964 |
| Sprague-Dawley rats | Dosing of ♂♀ via drinking water. High dose: 20 µg/mL until parturition, 10 µg/mL during weaning. Low dose: 20 µg/mL for 1 week, 40 µg/mL until parturition, 20 µg/mL during weaning. When dosed with highest concentration, mating proceeded, and litters were reduced to 8 pups. Litters from dosed ♂♀ either remained with original mother or were switched with control litter 1 d after delivery. All pups weaned and sacrificed on PND 20, 30 or 40. | Plasma LH analysis | • Prepubertal ♂ and ♀ offspring exposed to low dose of nicotine during lactation showed significant variation in LH levels from control  
• ♀ offspring of rats dosed during pregnancy or lactation showed significantly reduced body weight | Meyer and Carr, 1987 |
| Sprague-Dawley rats | Pregnant ♀♀ on gestation day 1 to implant subcutaneous minipump with 1.5 mg/kg/day saline or nicotine for 28 d. On PND 1, litter examined and saline-and nicotine-exposed pups cross-fostered to drug-free females. Maternal plasma levels of nicotine and cotinine (nicotine metabolite) determined after birth. Behavioral assessment with pups conducted on PND 5, 9, and 14. Striatal levels of neurotransmitter examined in 14 d pups. | Upon delivery: Number, viability, sex ratio, birth weight and body length  
Behavior: Position reflex, surface righting and negative geotaxis  
Biochemistry: DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC). | • Effective nicotine administration shown by nicotine and cotinine in maternal blood  
• Number of pups of nicotine treated ♀♀ reduced, as well as affecting pup body weight and length | Fung and Lau, 1989 |
| Sheep and Sprague-Dawley rats | Sheep: Pregnant ewes with ♀ fetuses fitted with catheters in fetal and maternal femoral veins on GD 130. After acclimatization, 10 or 25 µg/kg nicotine intravenously infused via the maternal vein in 5 min. Rat: From GD 3 to delivery, treated subcutaneously with | Sheep: Maternal and fetal heart rate and blood flow. Fetal blood analysis (pH, PO$_2$, PCO$_2$, lactic acid, hematocrit, Na$^+$ and K$^+$)  
Rat: Electrocardiogram in 4-5 mo ♀ rat offspring | • Fetal PO$_2$ decreased and PCO$_2$ increased with ewe dosing  
• Intravenous infusion of 10 and 25 µg/kg into ewes induced reduced heart rate within 15 min, followed by fetal heart rate | Feng et al., 2010 |
| Model organism | Methodology | Endpoint(s) assessed | Significant finding(s) | Reference |
|----------------|-------------|---------------------|------------------------|-----------|
| S-strain mice  | 5-15 d post mating, 0.1% aqueous solution nicotine injection (either subcutaneous or intraperitoneally) 1, 2 or 3 times (on consecutive days). Most were sacrificed at term, whilst some were sacrificed mid-pregnancy. | At term and mid-pregnancy observations: total litter, average litter, fetal death, congenital abnormalities | • Dosing induced fetal death and complete resorption at different time points of dosing (exposure at d 9, 10 and 11 most severely) | Nishimura and Nakai, 1958 |
| Swiss-Webster mice | ♀♀ dosed with nicotine for 5 weeks (dose increases as follows: Days 1 to 7 20 µg/mL; from day 8 60 µg/mL. For one group: from day 21 100 µg/mL). Breeding conducted after 2 weeks after final dosing. Pregnant ♀♀ were injected with 1.3 mg/kg nicotine either once or twice daily, from GD 12. On GD 17, mice sacrificed 20 min after receiving the final dose. | Morphology: Fetus and placenta weighed separately Biochemistry: α-aminoisobutyric acid (AIB) and acetylcholine (ACh) levels. | • Nicotine reduced fetal weight in concentration dependent manner • Dose-related inhibition of intracellular concentration of AIB when dosed via water • Nicotine injection 20 min prior to sacrifice induced similar intracellular AIB reduction, but not when injected 5 d prior to sacrifice | Rowell and Clark, 1982 |
| CD-1 mice       | 30-35 d old ♂ ♀ mice housed 6 per cage. Nicotine dissolved in 0.9% saline, injected intraperitoneally in doses of 0, 0.05, 0.4, or 0.8 mg/kg in 0.0075 mL/g 5, 15 or 25 min before assessment. Activity was simultaneously assessed as horizontal and vertical activity of two animals. | Activity: total distance moved, rest time, number of vertical/rearing movement, time response in open field activity, effect on striatal DA, ACh and carbohydrate metabolism | • 5-15 min after administration, 0.8 mg/kg reduced activity • 15-25 min after administration, 0.05 mg/kg increased activity by 28%, whereas 0.8 and 1.2 mg/kg reduced total distance by 56 and 77%, respectively; total distance decrease between 1.2 and 0.8 mg/kg different • Open field behavior affected by 0.8 mg/kg: depressant effect immediately set in, reached maximal effect 10 min after administration • Vertical rearing originally reduced by nicotine exposure but increased by 40 min • 0.8 mg/kg increased DOPAC levels • Glucose-specific activity and choline concentration reduced by 0.8 mg/kg in | Freeman et al., 1987 |
| Model organism          | Methodology                                                                 | Endpoint(s) assessed                                                                                           | Significant finding(s)                                                                                           | Reference                  |
|-------------------------|------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------|-----------------------------|
| **Thiacloprid**          |                                                                              |                                                                                                                |                                                                                                              |                             |
| Wildtype (AB) zebrafish  | Reared at 26°C and a 12 h light/dark cycle. FET test (OECD TG 236) and fish acute toxicity (AFT) test (OECD TG 203). Medium was renewed every 12 h. At 96 hpf, hatched larvae rinsed for biochemical and molecular analysis. Exposure concentrations: 438, 1750 and 7000 nM. | Toxicity testing Biochemical and molecular assays: MDA, total (T) GSH, oxidized glutathione (GSSG), ROS, CAT, T-SOD, Cu/Zn-SOD, peroxidase (POD), caspase 3, caspase 9, GST, CarE, and CYP450 | • Embryo LC50: 1.4 nM  
• Larval LC50: 2.86 nM  
• Juvenile LC50: 1.13 nM  
• Adult LC50: 2.97 nM  
• Exposure altered MDA, CAT, T-SOD, Cu/Zn-SOD, T-GSH, POD, Caspase3, ROS, CYP450, CarE, and GST levels  
• Relative mRNA levels of tsh, cyp19a, crh, Tnf, bax, p53, and cas8 affected | Wang et al., 2020 |
| Wildtype (WIK) zebrafish | Reared at 26°C, with a 14/10 h light/dark cycle. Eggs exposed to 1, 5, 10, 15, and 20 mg/L at 26, 28, 30 and 33.5°C. After 90 min, fertilized eggs transferred into fresh medium. At 26 and 28°C; experiments conducted until 96 hpf; remaining experiments ended at 72 hpf. Observations made at 8, 12, 24, 48, 60, 72, 84, and 96 hpf. | Morphology: mortality, heart rate, and development  
In situ hybridization: for ntl (10 hpf), krox20, and shh (13 hpf) | • Average heartbeat rate increased with temperature  
• Concentration-dependent transient increase of heartbeat rate followed by decrease at higher concentrations (peak at 10 mg/L) | Osterauer and Köhler, 2008 |
| **Thiamethoxam**         |                                                                              |                                                                                                                |                                                                                                              |                             |
| Wildtype (AB) zebrafish  | Reared at 28.5°C, on 14/10 h light/dark cycle. Treatment with 0.01, 0.1, 1, 10 and 100 mg/L; morphology studied at 3, 6, 10, 24, 72, and 96 hpf. Embryos exposed to 0.01 mg/L examined for surface tension effect from 0.75 to 24 hpf. Whole-mount in situ hybridization at 10 or 13 hpf. Behavioral analysis for 48 h from 4 dpf. | Morphology: survival, hatching, surface tension Behavior: swimming assay | • Embryo surface tension reduced compared to DMSO controls (DMSO slightly reduced surface tension compared to water controls)  
• Activity in the swimming assay overall reduced in a concentration-dependent manner | Liu et al., 2018 |

Ach, acetylcholine; AE, aerosol; AFT, acute fish toxicity test (OECD TG 203); AIB, α-aminoisobutyric acid; CarE, carboxylesterase; CAT, catalase; CYP450, cytochrome P450; DA, dopamine; DOPAC, 2,4-dihydroxyphenylacetic acid; ERC, environmentally relevant concentration; GD, gestation day; GSH, glutathione; GSSG, oxidized glutathione; GST, glutathione-S-transferase; HH, Hamburger-Hamilton; LDT, light-dark test; MDA, malondialdehyde; MO, Morpholinol antisense oligonucleotides; PND, post-natal day; POD, peroxide; PTU, 1-phenyl-2-thiourea; qRT-PCR, quantitative RT-PCR; ROS, reactive oxygen species; RT-PCR, real-time polymerase chain reaction; SOD, superoxide dismutase; T3, triiodothyronine; T4, thyroxine; TH, tyrosine hydroxylase; TS, tobacco smoke; VSRA, vibrational startle response assay; VTG, vitellogenin; WSC, worst case scenario concentration
Tab. S5: p-Values of the coiling assay replicates noted as statistically significant in Figures 1-4

Effects were rated statistically significant if at least 2 out of 3 replicates indicated statistical significance. In case replicates gave the same level of significance, the value is listed only once. For details of the statistical analysis, see Section 2.

| Mean burst duration |     |     |     | Mean burst count per minute |     |     |     |
|---------------------|-----|-----|-----|-----------------------------|-----|-----|-----|
|                     |     |     | p-value(s) |                                     |     |     |     |
| **Concentration**   |     |     |     | **Concentration**   |     |     |     |
| **Time point (hpf)**|     |     |     | **Time point (hpf)** |     |     |     |
|                     |     |     |     |     |     |     |     |
| 1.25                |     |     | <0.001, <0.01, <0.05 | 1.25 |     |     | <0.01, <0.05 |
| 23, 30, 45          |     |     |     | 23, 34, 40-47 |     |     |     |
| 23, 34, 40-47       |     |     |     | 24, 38, 39 |     |     | <0.01, <0.001 |
| 24, 38, 39          |     |     |     | 33 |     |     | <0.01, <0.001 |
| 27, 34, 45-47       |     |     |     | 36 |     |     | <0.01, <0.001 |
| 28, 39, 41, 46      |     |     |     | 37 |     |     | <0.001, <0.001 |
| 29, 31, 39-44, 47   |     |     |     | 50 |     |     | <0.001, <0.001 |
| 25                  |     |     |     | 24 |     |     | <0.001, <0.001 |
| 31                  |     |     | <0.05 | 33 |     |     | <0.001, <0.001 |
| 38                  |     |     | <0.05 | 36 |     |     | <0.001, <0.001 |
| 44                  |     |     | <0.05 | 37 |     |     | <0.001, <0.001 |
| 43, 44              |     |     | <0.05 | 38 |     |     | <0.001, <0.001 |
| 42, 44              |     |     | <0.05 | 39 |     |     | <0.001, <0.001 |
| 47                  |     |     | <0.05 | 40 |     |     | <0.001, <0.001 |
| 30, 44              |     |     | <0.05 | 41 |     |     | <0.001, <0.001 |
| 25                  |     |     | <0.05 | 42 |     |     | <0.001, <0.001 |
| 31                  |     |     | <0.05 | 43 |     |     | <0.001, <0.001 |
| 34                  |     |     | <0.05 | 44 |     |     | <0.001, <0.001 |
| 42, 45              |     |     | <0.05 | 45 |     |     | <0.001, <0.001 |
| 44, 46              |     |     | <0.05 | 43, 44 |     |     | <0.001, <0.001 |
| 38, 39, 41, 45      |     |     | <0.05 | 33 |     |     | <0.001, <0.001 |
| 36                  |     |     | <0.05 | 35 |     |     | <0.001, <0.001 |
| 24                  |     |     | <0.05 | 37 |     |     | <0.001, <0.001 |
| 23                  |     |     | <0.05 | 38 |     |     | <0.001, <0.001 |
| 25                  |     |     | <0.05 | 43, 44 |     |     | <0.001, <0.001 |
| 32, 34, 45-47       |     |     | <0.05 | 33 |     |     | <0.001, <0.001 |
| 25                  |     |     | <0.05 | 35 |     |     | <0.001, <0.001 |
| 33                  |     |     | <0.05 | 37 |     |     | <0.001, <0.001 |
| 23                  |     |     | <0.05 | 38 |     |     | <0.001, <0.001 |
| 24                  |     |     | <0.05 | 43, 44 |     |     | <0.001, <0.001 |
| 25                  |     |     | <0.05 | 47 |     |     | <0.001, <0.001 |
| 30                  |     |     | <0.05 | 44 |     |     | <0.001, <0.001 |
| 44                  |     |     | <0.05 | 31 |     |     | <0.001, <0.001 |
| 31                  |     |     | <0.05 | 38 |     |     | <0.001, <0.001 |
| 44                  |     |     | <0.05 | 47 |     |     | <0.001, <0.001 |
| 24                  |     |     | <0.05 | 32, 34, 45-47 |     |     | <0.001, <0.001 |
| 25                  |     |     | <0.05 | 27 |     |     | <0.001, <0.001 |
| 29                  |     |     | <0.05 | 29 |     |     | <0.001, <0.001 |
| 31                  |     |     | <0.05 | 38 |     |     | <0.001, <0.001 |
| 44                  |     |     | <0.05 | 44 |     |     | <0.001, <0.001 |
| 47                  |     |     | <0.05 | 40, 42 |     |     | <0.001, <0.001 |
| 38                  |     |     | <0.05 | 39 |     |     | <0.001, <0.001 |
| 45                  |     |     | <0.05 | 40 |     |     | <0.001, <0.001 |
| 44                  |     |     | <0.05 | 41 |     |     | <0.001, <0.001 |
| 46                  |     |     | <0.05 | 42 |     |     | <0.001, <0.001 |
| 38                  |     |     | <0.05 | 43 |     |     | <0.001, <0.001 |
| 45                  |     |     | <0.05 | 44 |     |     | <0.001, <0.001 |
| 46                  |     |     | <0.05 | 45 |     |     | <0.001, <0.001 |
| 38                  |     |     | <0.05 | 46 |     |     | <0.001, <0.001 |
| 47                  |     |     | <0.05 | 39 |     |     | <0.001, <0.001 |
| 38                  |     |     | <0.05 | 40 |     |     | <0.001, <0.001 |
| 46                  |     |     | <0.05 | 41 |     |     | <0.001, <0.001 |
| 45                  |     |     | <0.05 | 42 |     |     | <0.001, <0.001 |
| 44                  |     |     | <0.05 | 43 |     |     | <0.001, <0.001 |
| 38                  |     |     | <0.05 | 44 |     |     | <0.001, <0.001 |
| 37                  |     |     | <0.05 | 45 |     |     | <0.001, <0.001 |
| 38                  |     |     | <0.05 | 46 |     |     | <0.001, <0.001 |
| 47                  |     |     | <0.05 | 39 |     |     | <0.001, <0.001 |
| 38                  |     |     | <0.05 | 40 |     |     | <0.001, <0.001 |
| 46                  |     |     | <0.05 | 41 |     |     | <0.001, <0.001 |
| 38                  |     |     | <0.05 | 42 |     |     | <0.001, <0.001 |
| 38                  |     |     | <0.05 | 43 |     |     | <0.001, <0.001 |
| 38                  |     |     | <0.05 | 44 |     |     | <0.001, <0.001 |
| 38                  |     |     | <0.05 | 45 |     |     | <0.001, <0.001 |
| 38                  |     |     | <0.05 | 46 |     |     | <0.001, <0.001 |
### Tab. S6: p-Values of the swimming assay replicates after acetamiprid exposure (n = 2)

For details of the statistical analysis, see Section 2

| Distance moved (mm) | Mean burst count per minute |
|--------------------|-----------------------------|
| Concentration      | p-value(s)                  | Concentration | p-value(s) |
| Acetamiprid 100 µM | <0.01, <0.05                | 100 µM        | <0.05      |
| 110-114            | <0.001, <0.05               |              |            |

### Tab. 7: ANOVA results for the analysis of the swimming assay total distance swam by zebrafish (Danio rerio) embryos exposed to acetamiprid or nicotine throughout the entire recording duration

Acetamiprid: n = 2; nicotine: n = 3 (19 individuals per treatment group per replicate). For details on statistical analysis, see Section 2

| Replicate | Difference to 0.1% DMSO | Lower 95% CI | Upper 95% CI | Std. dev | t-Value | p-Value |
|-----------|--------------------------|--------------|--------------|----------|---------|---------|
| Acetamiprid 50 µM | 1 -93.42                 | -177.62      | -9.21        | 33.88    | -2.76   | 0.0247  |
|           | 2 -54.53                 | -107.22      | -1.84        | 21.20    | -2.57   | 0.0401  |
|           | Ø -74.00                 | -123.73      | -24.27       | 20.01    | -3.70   | 0.0112  |
|           | 1 -144.23                | -228.44      | -60.03       | 33.88    | -4.26   | 0.0002  |
|           | 2 -93.32                 | -145.93      | -40.54       | 21.20    | -4.30   | 0.0001  |
|           | Ø -118.76                | -168.49      | -69.03       | 20.01    | -5.94   | 6.90^6  |
| Acetamiprid 100 µM | 1 -118.76                | -168.49      | -69.03       | 20.01    | -5.94   | 6.90^6  |
|           | 2 -93.32                 | -145.93      | -40.54       | 21.20    | -4.30   | 0.0001  |
|           | Ø -118.76                | -168.49      | -69.03       | 20.01    | -5.94   | 6.90^6  |
| Nicotine 2.5 µM | 1 108.53                 | 8.95         | 208.11       | 40.06    | 2.71    | 0.0282  |
|           | Ø 53.86                  | 2.40         | 105.33       | 20.7     | 2.60    | 0.0372  |
| Nicotine 12.5 µM | 1 60.40                  | 8.93         | 111.86       | 20.7     | 2.92    | 0.0159  |
|           | Ø 50.64                  | 8.17         | 111.10       | 20.7     | 2.88    | 0.0176  |

CI, confidence interval; Std. dev, standard deviation
Tab. S8: Detailed list of observations made in FET tests after 24, 48, 72, 96 and 120 exposure and to 6.25 (1), 12.5 (2), 25 (3), 50 (4) and 100 µM (5) of the neonicotinoids and nicotine

| Endpoint                                | Developmental time-point | 24 hpf | 48 hpf | 72 hpf | 96 hpf | 120 hpf |
|-----------------------------------------|--------------------------|--------|--------|--------|--------|---------|
| Concentration                           |                          | 1      | 2      | 3      | 4      | 5       | 1      | 2      | 3      | 4      | 5       |
| Spontaneous movement (↓, ↓↓)            |                          | N      | N      | N      | N      | N       | N      | N      | N      | N      | N       |
| Spontaneous movement (↑)                |                          | A I N  | A I N  | A D I  | A D I  | TC TM   | A D I  | A D I  | A D I  | A D I  | A D I  |
| Delayed hatching                        |                          |        |        |        |        |         |        |        |        |        |         |
| Heartbeat (↓, ↓↓, ↓↓↓)                  |                          | I N    | A A    | TM     | I A    | A N N   | A A    | A A    | A A    | A I N  | A A A A |
| Blood flow (↓, ↓↓, ↓↓↓)                 |                          | I A A   | A TM I | A N N   | A A I  | A A A   | A I N  | A A    | A A    | A A    | A A    |
| Spinal deformation (K, L)               |                          |        |        |        |        |         |        |        |        |        |         |
| Reduced body length                     |                          | A C    | A C    | A C D   | A C D   | A C D   | A C D   | A C D   | A C D   | A C D   | A C D   |
| Edema                                   |                          | N I    | A A    | N A A   | A N N   | A N N   | A N N   | A N N   | A N N   | A N N   | A N N   |
| Otolith deformation                     |                          |        |        |        |        |         |        |        |        |        |         |
| Pigmentation (↓, ↓↓, ↓↓↓)               |                          | A C D   | A C D   | A C D   | A C D   | A C D   | A C D   | A C D   | A C D   | A C D   | A C D   |
| Pericardial inflation                   |                          | A A A   | A A A   | A A A   | A A A   | A A A   | A A A   | A A A   | A A A   | A A A   | A A A   |
| Craniofacial deformation                |                          |        |        |        |        |         |        |        |        |        |         |
| Reduced yolk resorption                 |                          | N N A   | N A N   | N N N   | N N N   | N N N   | N N N   | N N N   | N N N   | N N N   | N N N   |
| Tremor/twitching                        |                          |        |        |        |        |         |        |        |        |        |         |
| Increased late activity                 |                          |        |        |        |        |         |        |        |        |        |         |

1-5: lowest to highest exposure concentrations: 6.25, 12.5, 25, 50 and 100 µM; 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 in blue: time points during which this endpoint cannot be observed.
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