von Hellfeld et al.:

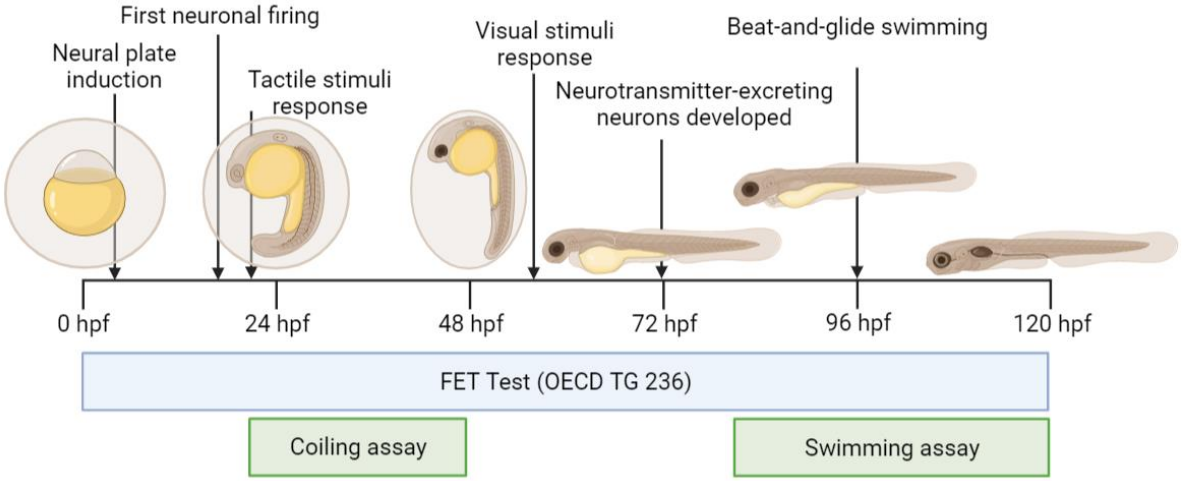
Zebrafish Embryo Neonicotinoid Developmental Neurotoxicity in the FET Test and Behavioral Assays

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

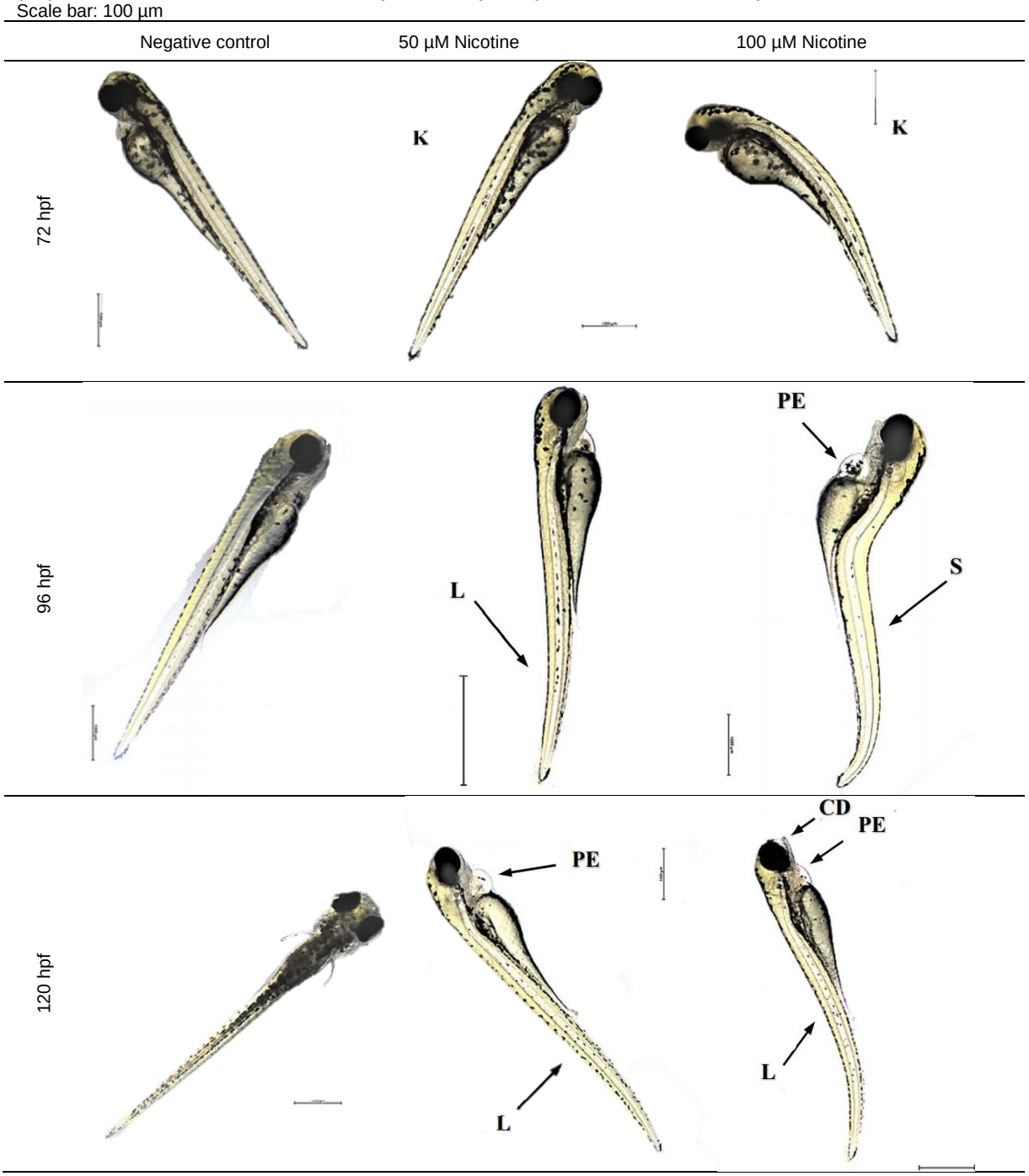
Fig. S1: Overview of the neurogenesis of zebrafish embryos, as the basis of behavioral assays such as the coiling and the swimming assay
Figure created with biorender.com



doi:10.14573/altex.2111021s1

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



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[a]	CAS no.	Chemical class	Log K_{ow}[b]	Water solubility[b]	Stability and biodegradability[c]
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
Thiacloprid	252.72	111988-49-9	Chloropyridyl	1.26	185 mg/L	Half-life time: 10-63 d
Thiamethoxam	291.71	153719-23-4	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; [a] Sigma Aldrich: https://www.sigmaaldrich.com; [b] PubChem: https://pubchem.ncbi.nlm.nih.gov; [c] 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	0.3
Dinotefuran	202.21	203.05>129.03	14	10	ESP+	Formic_FAST_B2	0.75
Imidacloprid	255.66	256.99>175.97	14	20	ESP+	Formic_FAST_B2	0.75
Nicotine	162.23	163.06>129.99	42	20	ESP+	Acetate_FAST_B2	0.3
Thiacloprid	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

Tab. S3: Camera configuration and recording settings of the EthioVision®XT software for the coiling and swimming assays

	Setting	Parameter
Coiling assay	**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	**Video settings**	
	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	160
	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
Acetamiprid				
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 LC_{50}: 518 mg/L • 120 hpf EC_{50}: 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 • Tail and head touch less sensitive; head touch response being more pronounced than tail touch at 974 mg/L	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 anto-CD206	• 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	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) • Aggression level in low-dose group ♂♂ significantly increased in total duration and number of bouts compared to high-dose	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 Immunohistochemistry: plasma testosterone	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)				
A/J mice	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, Cy11a1,* and *Hsd17b1*	Terayama et al., 2016
Acetamiprid, clothianidin, dinotefuran and thiamethoxam				
Wildtype zebrafish	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 habituation significantly reduced. At WSC, startle response increased, habitation reduced • Clothianidin: At WSC, startle response increased • Dinotefuran: At ERC and WSC, habituation reduced	Faria et al., 2020
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. *In vivo* mice: 5-6 wks ♀♀ mated and fed with 0.3 or 3 mg/kg bodyweight thiacloprid *via* feed and syringe; ♀♀ sacrificed after 80 h dosing and embryos isolated.	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	Babeľová et al., 2017
Clothianidin				
ICR mice	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
	divided into 4 groups, collecting blood from dams and fetuses at 1, 3, and 6 h after administration on GD 18, after which animals were sacrificed. The daily dose group received clothianidin once a day from GD 10 and 15 to 18; samples taken 6 h after dosing.		than in 3 and 6 h animals. Concentrations of metabolites increased with increasing time after administration • Positive correlation between blood levels of maternal clothianidin and that of the offspring (85% that of the dams); similar findings for metabolites	
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 *grin1, 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
Dinotefuran				
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
C75BL/6NrSlc mice	3 wks ♂mice dosed with 0, 100, 500, and 2500 mg/kg/day dinotefuran for 5 wks. Body weight measured twice a week. On final dosing day, behavioral assays; sacrifices performed the following day.	Morphology: Body weight Behavior: Tail suspension test and forced swim test	• In the tail suspension test, animals dosed 500 mg/kg/day less immobile than controls	Takada et al., 2018
Dinotefuran & imidacloprid				
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: *CU7Zn-sod, Mn-Sod, cat, gpx1, glcl, cyp1a, gstm, gtp1, ugt1a,* and *ß-actin* Genotoxicity: comet assay	Dinotefuran: • 0.1 mg/L reduced *Cu/zn-sod* and *gstm* expression, 0.5 mg/L reduced *cat, cyp1a*, and *gstp1.* 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
Imidacloprid				
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.	• 48 hpf LC_{50}: 186.9 mg/L • 96 hpf LC_{50}: 143.7 mg/L	Wang et al., 2017
Wildtype (AB) zebrafish	Reared at 26°C, 14/10 h light/dark cycle. Fertilized eggs (2 hpf), larvae (72 hpf), 1 mo juveniles and 3 mo adults utilized for multiple life-stage assays. For biochemical analysis, embryos exposed until 96 hpf.	Morphology: Mortality, LC, development. 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 LC_{50}:121.6 mg/L • Larval LC_{50}:128.9 mg/L • Juvenile LC_{50}:1.13 26.39 mg/L • Adult LC_{50}: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 *tsh* mRNA levels, increased *Cu/Zn-sod, bcl-2, ucp-2, bax, p53*, and *TRß* mRNA levels	Wu et al., 2018

Model organism	Methodology	Endpoint(s) assessed	Significant finding(s)	Reference
		TRß, Dio1, Dio2, and *tsh*		
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	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: i*n situ* for *vhmc, 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 n*kx2.5* 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	Reared at 28.5°C on 14/10 h light/dark cycle. 2 hpf eggs placed in 50 mL aqueous solution with 35 individuals per dish for 5 d with daily medium renewal. Exposure to 45 or 60 µM nicotine or imidacloprid. At 5 dpf, embryos transferred to un-dosed aquarium water. Embryos for assessment placed in	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
	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 during 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	
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: VSRA, habituation	• Nicotine-exposed larvae moved more during the VSRA • > 25 µM imidacloprid reduced distance moved in a concentration-dependent manner	Faria et al., 2019
Nicotine				
Wildtype zebrafish	Rearing in 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_{50}: 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 *bcrn3c:*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
		Ultrastructural changes: scanning electron microscopy		
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.	Morphology (at < 66 hpf): body length Behavior: escape and swimming on tactile stimulus of tail bud Nervous system development: immunocytochemistry, GFP visualization and motoneuron innervation assessment	• 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) Immunohistochemistry: [^{3}H]-nicotine uptake: measuring –(-)-[N-methyl-^{3}H] 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
			water concentration	
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 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 *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) Immunohistochemistry: zn8 (aka. zn5), znp1, F59, and zn12	• α2A nAChR mRNA present in olfactory neurons and spinal cord from 19 hpf (probably Rohon beard neurons) • Translated protein also found present in olfactory epithelium, spinal cord, and muscle • 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* • α2A morphants showed reduced bend rates immediately after exposure to 60 µM nicotine • 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 • α2A MO did not disrupt formation of muscle-specific nAChRs • 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 Live imaging of *isl1* embryos Morphology: *via* whole-mount immunohistochemistry (F59, F310, znp1, zn5, and anti-ß2), and histology	• Exposed embryos had shortened dorsal/ventral axis with disorganized atrophic muscles • Nicotine altered slow and fast muscles in wildtype and *isl1* embryos • 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, ß2, α7, α3 and ß4. 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 qPCR: *ß1a, ß1b*, and *Elongation factor1-α* (*Elf1- α*)	• All tested receptors responded well to > 3 µM acetylcholine • Nicotine partial agonist for all heteromeric receptor subtypes, being most potent for α4ß2 and least potent for muscle-type receptors • 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 Gene expression at 24 hpf	*In vivo:* • 34 µM TS extract reduced survival at 24 hpf • 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*) *In vitro:* • TS extract > 6.8 µM altered beats per minute and cardiomyocyte maturity, whilst 13.7 µM reduced cardiomyocyte yield and purity • TS affected gene expression more significantly than EA extracts	
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	Experiment 1: • Significant reduction in fetal body weight • Brain weight slightly increased, liver weight not affected • Significant differences between treated and control rats in liver lipid/tissue, lipid/nitrogen, and cholesterol/lipid ratios Experiment 2: • Nicotine during weaning led to rougher fur and reduced fetal mean body weight	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	Sheep: • 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
	either 0.3 mL saline solution or 1.5 mg/kg nicotine hydrogen tartrate twice daily. Pups born naturally and allowed to wean. ♂ offspring removed after weaning and examined at 4-5 months of age, assessing heart rate of control and exposure groups after acclimatization and after injection of 2 mg/kg nicotine.		increase • Various types of fetal arrythmia only after maternal nicotine infusion Rat: • Heart rate of nicotine exposed rats higher during immobilization period • After nicotine injection, rats of un-dosed and dosed maternal rats showed decreased heart rate • Offspring of exposed rats showed less of a decrease • Nicotine injection increased arrythmia in exposed offspring more than in control offspring	
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) • Most malformations linked to the skeletal system, predominantly affecting the limbs, as well as spinal curvature and cleft palate	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
			striatum, hippocampus, and cortex (no effect on acetylcholine)	
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 LC_{50}:1.4 nM • Larval LC_{50}:2.86 nM • Juvenile LC_{50}:1.13 nM • Adult LC_{50}: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	• 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 *In situ* hybridization: for *ntl* (10 hpf), *krox20,* and *shh* (13 hpf) 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, Morpholino 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		
	Concentration	**Time point (hpf)**	***p*-value(s)**	**Concentration**	**Time point (hpf)**	***p*-value(s)**
Nicotine	1.25	39, 40	<0.001, <0.01, <0.05	1.25	30, 34	<0.01, <0.05
		41-45	<0.001, <0.01		36	<0.01
					37, 38, 40-42, 47	<0.001
					39, 43, 46	<0.001, <0.05
					44, 45	<0.001, <0.01
	2.5	23	<0.001, <0.01	2.5	23, 25, 40-47	<0.001
		24	<0.001, <0.05		24, 38, 39	<0.001, <0.01
		28, 38, 39, 41, 46	<0.01, <0.05		33	<0.01, <0.05
		40, 45	<0.01		35	<0.05
		42-44	<0.001, <0.01, <0.05		37	<0.001, <0.01, <0.05
	12.5	23, 30, 38, 45, 46	<0.001, <0.01	12.5	23-26	<0.001
		24-29, 31, 39-44, 47	<0.001		27, 34, 45-47	<0.001, <0.01
					33	<0.01
					35, 44	<0.001, <0.05
					36	<0.001, <0.01, <0.05
					37	<0.05
	25	23-47	<0.001	25	23, 40	<0.001, <0.05
					24-28	<0.001
					29-33	<0.001, <0.01
					34	<0.001, <0.01, <0.05
					40-42	<0.01, <0.05
					43, 44	<0.05
	25	31	<0.05	25	23	<0.001
		39	<0.05		24	<0.01, <0.001
		40	<0.001		33	<0.05
					38, 39, 43, 45	<0.05
					40, 42	<0.01
					44, 46	0.001
	50	40-42	<0.05	50	23	<0.01, <0.001
		44	<0.01		24	<0.001
					42, 45	<0.05
					44, 46	<0.001
	100	38	<0.001	100	23	<0.001, <0.05
		39-41	<0.001		24	<0.001, <0.01, <0.05
		42, 44	<0.01		25	<0.01
		45	<0.05		33, 34	<0.001, <0,05
					44	<0.01
Imidacloprid*	2.5	24	<0.001	2.5	24	<0.001, <0.01
		28	<0.01, <0.05		25	<0.001
		29	<0.001, <0.01		47	<0.01
	25	24	<0.001, < 0.05	25	25	<0.001
		25	<0.05, < 0.001		27	<0.001, <0.05
		27, 35	<0.01, <0.05		29	<0.05
		29	<0.001, <0.05		38	<0.01
					44, 45	<0.05
					47	<0.001
	50	24	<0.001, <0.01, <0.05	50	24	<0.01
		40	<0.01		31	<0.01, <0.05
					38	<0.01
					44	<0.05
					47	<0.001
	100	24	<0.001, <0.01	100	24	<0.001
		25	<0.001, <0.01		31	<0.001, <0.05
		29	<0.001, <0.05		38	<0.01

	Mean burst duration			Mean burst count per minute		
	Concentration	**Time point (hpf)**	***p*-value(s)**	**Concentration**	**Time point (hpf)**	***p*-value(s)**
		38, 39, 43, 46	<0.01		40	<0.05
		40, 44, 45, 37	<0.001		41	<0.001
		41	<0.05			
Thiacloprid	2.5	35	<0.01, <0.05			
		42	<0.05			
	25	39	<0.05			
		41	<0.001, <0.05			
	50	38	<0.05	50	24	<0.001, <0.05
		39, 41	<0.001, <0.05			
		40	<0.001			
		42	<0.001, <0.01			
		43	<0.001, <0.01, <0.05			
		44	<0.01, <0.05			
	100	35, 46	<0.001, <0.05	100	24	<0.05
		37	<0.05			
		38, 41, 42	<0.001			
		39, 40, 43	<0.001, <0.01			
		44	<0.01			
		45, 47	<0.01, <0.05			

*, Compounds, where significant values after 37 hpf were based on one replicate; blue highlight: values based on 1 replicate at the post-37 hpf time point (acetamiprid and imidacloprid only).

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	**Time period (hpf)**	***p*-value(s)**	**Concentration**	**Time period (hpf)**	***p*-value(s)**
Acetamiprid	100 µM	100-104	<0.01, <0.05	100 µM	110-114	<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
	100 µM	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^{-06}
Nicotine	2.5 µM	2	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
	12.5 µM	Ø	60.40	8.93	111.86	20.7	2.92	0.0159
	25 µM	3	96.86	2.40	105.33	20.7	2.60	0.0372
		Ø	59.64	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	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Spontaneous movement (↓, ↓↓)	N	N	N	N	N																				
Spontaneous movement (↑)	AIN	AIN	ADI	ADITC	ADITCTM																				
Delayed hatching																	N	TM		N					
Heartbeat (↓, ↓↓, ↓↓↓)						IN	A	A	TM									C	A					N	
Blood flow (↓, ↓↓, ↓↓↓)						I	A	A	TM	I			A	N	N	A	AI	A	A	AIN			A	AN	AN
Spinal deformation (K, L)																									
Reduced body length											AC	ACTM	ACDNTM	CDNTM	ACDNTM	AN	AN	CNTM	IN	NTM	A	AN	A	AN	AN
Edema														N	I				AN	A				N	N
Otolith deformation																									N
Pigmentation (↓, ↓↓, ↓↓↓)						ACDINTCTM	ACDINTCTM	ACINTCTM	ACDINTCTM	ACDINTCTM	ACNTM	ACDNTM	ACDNTCTM	ADNTM	ADN	I	I	AI	IN	IN					
Pericardial inflation							A	A	A	A	A	A	A	A	A	A	A	A	A	A	A		A		
Craniofacial deformation															N				N	AN		N		N	N
Reduced yolk resorption																			N	N				N	N
Tremor/twitching																								N	N
Increased late activity																TC		TC	TCTM	TCTM	ANTCTM	ACNTCTM	ANTCTM	ACDTCTM	ACDTCTM

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.

References

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