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Imidacloprid induces adverse effects on fish early life stages that are more severe in Japanese medaka (*Oryzias latipes*) than in zebrafish (*Danio rerio*)

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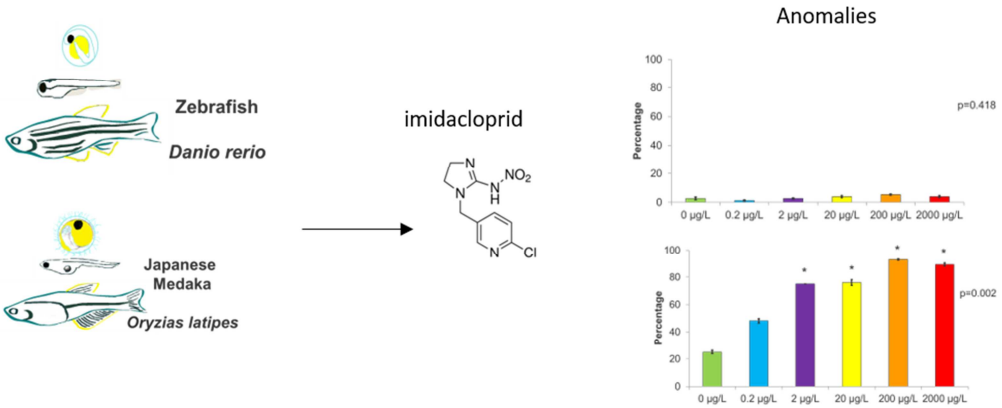
ABSTRACT

Neonicotinoids are widely used insecticides that have frequently been found in freshwater with concentrations ranging from ng to µg/L. It is known that these compounds impact non-target invertebrates, such as bees and gammaridae, in terms of toxicity and behavior, but impacts and species differences on vertebrates such as fish are little explored. The aim of this study was to investigate and compare the effects of one widely used neonicotinoid, imidacloprid, on development and behavior of two fish model species: Zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*). Fish were exposed for 5 (zebrafish) and 14 (medaka) days from 0.2 to 2000 µg/L imidacloprid by aqueous exposure. Survival, development, behavior and histological features were monitored and organism-internal concentrations and biotransformation products measured. Imidacloprid caused sublethal effects in both species but the effects were much stronger in medaka with deformities, lesions and reduced growth being the most prominent impacts. Due to the overall longer time of development, time-integrated exposure of medaka was about 2-fold higher compared to zebrafish, potentially accounting for parts of the sensitivity differences. Our results underline the importance of taking species sensitivity differences into account especially when considering that medaka responded at imidacloprid concentrations that have been measured in the environment.

KEYWORDS: Cyprinids, toxicokinetics, species sensitivity, metabolome, embryo toxicity, imidacloprid.

HIGHLIGHTS:

- Imidacloprid impacts on fish at environmentally relevant concentrations.
- At the same developmental stage, medaka are more sensitive than zebrafish.
- Our study supports the importance of taking species sensitivity differences into account



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1. INTRODUCTION

Neonicotinoids are one of the most produced pesticide families, even after the partial ban in Europe since 2013 (Van Dijk *et al.* 2013, Simon-Delso *et al.* 2014, Bonmatin *et al.* 2015, Wood *et al.* 2017). They are low molecular weight and highly hydrophilic insecticidal chemicals that are applied in agriculture in various ways, including foliar sprays and seed treatments (Bonmatin *et al.* 2015). The intended mode of action of these molecules is to bind to nicotine acetylcholine receptors (nAChR) in nervous tissues in insects, causing dysregulation of neurotransmission at cholinergic synapsis, which can lead to overstimulation, tremors, paralysis and death (Sánchez-Bayo 2012, Simon-Delso *et al.* 2014). Yet, based on this mode of action, neonicotinoids could affect signal transmission and behavior of other organisms with a developed neuronal system (Sánchez-Bayo 2012). These include animals living in aquatic environments (Tennekes 2011, Sánchez-Bayo 2012, Roessink *et al.* 2013, Sánchez-Bayo 2014).

Imidacloprid is one of frequently detected and well-studied neonicotinoids. It has been detected up to several hundred µg/L after agricultural use but has most commonly been found in the low ng/L range in continental water bodies (Moschet *et al.* 2014). Morissey et al. (2015) reported up to 320 µg/L imidacloprid in drainage ditches in the Netherlands while Anderson et al.(2015) documented a peak concentration of 0.7 µg/L in a Canadian surface water over a general background concentration of 0.04 to 0.05 µg/L. As demonstrated by aquatic species sensitivity distribution on survival after a few days of exposure (SI Figure S1), insects are the most vulnerable organism group, followed by crustaceans, while fish appear several orders of magnitude less sensitive to direct short-term exposure of imidacloprid. Indirect effects on fish, such as a loss of the quantity and quality of crustaceans serving as food (Hayasaka *et al.* 2012a, Gibbons *et al.* 2014, Chagnon *et al.* 2015), have been proposed. However, direct sub-lethal effects on fish, especially during early developmental stages, have rarely been explored. Reduced locomotion was reported in zebrafish larvae continuously exposed to imidacloprid from fertilization to five days (Crosby

et al. 2015). No impact was reported for zebrafish development when exposed to imidacloprid from fertilization to 48 hours (Tišler *et al.* 2009) and 96 hours (Scheil *et al.* 2009) of development, whereas growth of medaka adults and juveniles was reduced after long term exposure in mesocosms (Hayasaka *et al.* 2012b). Another study showed stress syndrome in medaka juvenile and increase parasite infestation after exposure to imidacloprid (Sanchez-Bayo *et al.* 2005). All these studies used exposure concentrations in the mg/L range, which is much higher than the concentrations found in the environment.

The aim of our study was to test if direct sub-lethal effects can be elicited by imidacloprid at concentrations that include environmentally realistic exposure levels during critical stages of development, i.e. early life, in model fish species: Zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*). While both species share common features such as large broods, breeding all year and transparent eggs that develop outside the mother, a distinct difference is their time of development (SI Figure S2) (Kimmel *et al.* 1995, Furutani-Seiki *et al.* 2004, Iwamatsu 2004). While zebrafish hatch after 3 days post fertilization (dpf), medaka require an average of 9 dpf to emerge as free-swimming larvae. Thereafter, free swimming larvae are completely established within 5 dpf in zebrafish where it takes 14 dpf in medaka. We thus hypothesized that potential sub-lethal effects would be stronger in medaka because of the longer developmental time and consequently greater time-integrated exposure. To test this hypothesis, internal imidacloprid concentrations and physiological and histological alterations were examined for both species at similar developmental stages. Medaka indeed was more severely affected than zebrafish by imidacloprid exposure which led us to explore the relative abundance of metabolites involved in energy metabolism and neurotransmission in this fish.

2. MATERIAL AND METHODS

2.1 Embryo collection

Zebrafish wild type (WT) were maintained and bred on site at the Eawag facility according to the guidelines published by Nusslein-Volhard and Dahm, 2002 (Nüsslein-Volhard *et al.* 2002). Fish were raised at 28℃ in 14/10h light/dar k cycle in reconstituted water (294.0 mg/L $CaCl_2$, $2H_2O$, 123.2 mg/L $MgSO_4$, $7H_2O$, 64.74 mg/L $NaHCO_3$ and 5.7 mg/L KCl; prepared in MilliQ water, pH 7.5) and fed twice daily with a combination of live food (*Artemia nauplia*) and dry flakes (Tetramin, Switzerland). Adult fish were maintained in a large breeding tank (Aquatic habitat) with a special spawning system for collecting eggs. Eggs were collected between 1 and 2 hours after the lights were turned on and fertilized eggs separated from unfertilized and placed in fresh medium in Petri dishes. Only spawns with more than 80% of fertilized eggs were kept for the exposure. Medaka embryos were ordered from AMAGEN platform in Gif-sur-Yvette (France). They were transferred to the laboratory in hermetic boxes and immediately used with exposure starting at 13 hpf. All procedures were in accordance with the animal protection guidelines. Experiments with zebrafish and medaka larvae were approved by the Swiss Cantonal Veterinary Office (Number 119/2014) and by the French ethic committee (Number A33-522-7), respectively.

2.2 Imidacloprid exposure

Based on concentrations reported from different water environments, the imidacloprid exposure range was set from 0.2 to 2000 µg/L. Imidacloprid (PESTANAL®, analytical standard, Sigma-Aldrich 37894) was prepared as a 200 mg/L stock solution in 250 mL of reconstituted water and aliquoted before being stored at -20℃. Serial dilutions with a factor of 10 were prepared daily from this stock solution (see below). Three times twenty-five fertilized embryos were placed in 3.5 cm Petri dishes with 3 mL of reconstituted water without (control) or with imidacloprid. Exposure lasted for 5 (zebrafish) and 14 (medaka) days post fertilization (dpf). Water exchange was done every 24 hours to ensure stable aqueous imidacloprid exposure concentrations (see chemical analysis below). Zebrafish were raised

at 28℃ and medaka at 26℃ in incubators (Economic Delux ECD01E model, Snijders Scientific, Tilburg, NL for zebrafish and Memmert ICP 700 for medaka) in 14/10h light/dark cycle in reconstituted water.

2.3 Survival and development

Survival was monitored daily under the microscope and dead embryos or larvae were removed. Fish were considered as dead when the heart did no longer beat. Percent survival was calculated as compared to control for the last time point by using the ratio of survival divided by the initial number of embryos.

Hatch was likewise monitored daily. In controls, it is expected to occur around 3 days post fertilization (dpf) for zebrafish (Kimmel *et al.* 1995) and 9 dpf for medaka (Iwamatsu 2004). Hatchability was expressed as percent of control and calculated using the ratio of hatching larvae divided by the initial number of embryos.

After hatching (at 3 dpf for zebrafish and 9 dpf for medaka), 10 larvae per replicate and per treatment were individually placed in 96 well plates. Microscope images of the whole body of each larvae were taken for length measurement. Size was measured from mouth to end of the tail. 10 larvae per biological replicate (3 replicates) were analyzed for a total of 30 fish per treatment.

Developmental anomalies were analyzed at the same time as size measurements under the microscope following the protocol published by Le Bihanic (2013). Different types of anomalies were recorded: heart, yolk-sac or bone oedema, tail problems (lordosis, kyphosis or scoliosis), jaw or skull deformity, ocular lesions (missing eye, cyclopia and dystrophy), heart curvature/position, hemorrhage and presence or absence of swollen swim bladder. These results were expressed as percent compared to unexposed control.

2.4 Behavior

Behavioral experiments were performed at 5 dpf with zebrafish larvae (using Zebrabox from Viewpoint) and at 14 dpf with medaka larvae (using Daniovision from Noldus EthovisionXT11) and distance moved was video-tracked in both cases. First, fish were acclimatized in well plates in the dark for 2 hours at their optimal temperature (26℃ for medaka and 28℃ for zebrafish) before the test and then recorded for 3 periods. The first one was in the dark (light off-1; L.off-1), the second one in light (light on; L.on) and the third one in dark (light off-2; L.off-2).

For zebrafish, the procedure was as previously described ((Vignet *et al.* 2013, Vignet *et al.* 2015). The tested plate was transferred into the Zebrabox. Then 12 randommly selected zebrafish larvae per replicate per treatment were tested in 24 well plates in 5 min intervals. In light-off periods, zebrafish larvae normally present an increase of activity.

The procedure for medaka was as previously described in Granger Joly de Boissel *et al.* (2017). 10 larvae per replicate were randomly selected and placed individually in wells of a 48 well plate. After acclimatization, medaka larvae were video tracked in 10 min intervals. The last Light off (L.off-2) period represents the peak activity for medaka. According to Le Bihanic (2014) and Chiffre (2016), medaka activity is constant during the L.off-1 period and slightly increases during the L.on whereas for the last period, when the light is turned off again, larvae react with an increase of activity.

2.5 Histology

Eight samples for histological assessment were collected from control and each treatment at 5 dpf for zebrafish and at 14 dpf for medaka. The larvae were anesthetized with 0.01% MS 222 (Tricaine Methanesulfonate), and then immediately fixed in the Surgipath Decalcifier (Leica) for 24 h at 4℃. After dehydration in ethanol, all specimens were embedded in paraffin (Bio-Optica, Italy) and sectioned at a thickness of 5 µm with a rotary automatic

microtome (Leica Microsystems, Wetzlar, Germany). Serial sections were stained with hematoxylin and eosin (Bio-Optica, Isttaly), and examined with a motorized Zeiss Axio Imager Z1 light microscope (Carl Zeiss AG, Werk Göttingen, Germany), equipped with an AxioCam digital camera (Zeiss, Jena, Germany) for the acquisition of images. This protocol was already published (Fasulo *et al.* 2010, Maisano *et al.* 2016, Maisano *et al.* 2017)

2.6 Liquid chromatography–high resolution mass spectrometry (LC-HRMS) analysis of imidacloprid and its biotransformation products in fish and exposure medium

Internal concentrations of imidacloprid and its biotransformation products were determined in a separate set of experiments. A pool of 150 eggs was used in each of three independent experiments in which fish were raised up to 5 dpf for zebrafish and up to 14 dpf for medaka in two Petri dishes with 20 mL of reconstituted water in each with 0 or 2000 µg/L of imidacloprid. Zebrafish were sampled after fertilization and at 3 dpf (hatching day) and 5 dpf (larval stage and end of the exposure) while medaka were sampled after fertilization, at 3 dpf (same exposure days as zebrafish), 5 dpf (same exposure day as zebrafish), 9 dpf (hatching day) and 14 dpf (larval stage and end of the exposure). The 150 embryos or larvae (depending on the stage) per treatment were transferred into a cryotube and rinsed three times with nanopure water. Water was removed with a pipet as much as possible and tubes were weighted and immediately flash-frozen in liquid nitrogen.

The sample preparation was based on the method by Rosch et al. (2017). In general, 100 µL of 100 µg/L imidacloprid_d_4, 500 µL MeOH, and 300 mg of 1 mm zirconia/silica beads (BioSpec Products, Inc., U.S.A.) were added to the frozen organisms, followed by homogenization and extraction using a FastPrep bead beater (MP Biomedicals, Switzerland) in two cycles (15 s, 6 m/s). The homogenized samples were centrifuged (6 min, 10 000 rpm, 20 ℃) and filtered through 0.45 µm regenerated cellulose filters (BGB Analytic AG, Switzerland). The supernatant was collected and the filters were washed with 400 µL MeOH. The filtrate and the extract were eventually combined.

Imidacloprid concentration in embryo exposure medium was monitoring in the daily changed solution and was sampled over the exposure to measure the effective imidacloprid concentration (SI Table S1). All samples were stored at −20 ℃ until chemical analysis. The samples were analyzed by online solid phase extraction coupled to reversed phase liquid chromatography high resolution tandem mass spectrometry (online-SPE-LC-HRMS/MS) (Q Exactive, Thermo Fisher Scientific Inc.). Detection was done by full scan acquisition with a resolution of 70000 (at m/z 200) in polarity switching mode (electrospray ionization) followed by five (positive mode) and two (negative mode) data-dependent MS/MS scans with a resolution of 17000 (at m/z 200) with an isolation window of 1 m/z. Quantification was carried out for imidacloprid and its metabolites using standards and imidacloprid-d4 as internal standard.

2.7 NMR-based metabolomics analysis in medaka larvae

Endogenous polar metabolites from 14 dpf medaka larvae (n=3 pools of 20 fish each per group) were extracted using a “two-step” methanol/chloroform/water protocol, adequately modified (Wu *et al.* 2008, Cappello *et al.* 2017b). In brief, medaka larvae were homogenized in 8 mL/g of cold methanol and 2.5 mL/g of cold water by a TissueLyser LT bead mill (Qiagen) with 0.5 mm glass beads, for 5 min at 50 vibrations/s, twice. Homogenates were transferred into glass vials, and 8 mL/g chloroform and 4 mL/g water were added. Samples were vortexed for 30 s, and then incubated on ice for 10 min for phase separation. Following centrifugation at 2000 g for 5 min at 4 ℃, 200 µL of the upper methanol layer were transferred into glass vials, dried in a centrifugal vacuum concentrator (Eppendorf 5301), and kept at -80℃. Prior to Nuclear Magnetic Resonance (NMR) analysis, the dried polar extracts were resuspended in 600 µL of a 0.1 M sodium phosphate buffer (pH 7.0, 10% D_2O (Armar AG, Döttingen, Switzerland)) containing 1 mM 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) (Sigma-Aldrich Co) used as internal standard, and then pipetted into a 5 mm NMR tube.

Extracts of medaka larvae were analyzed on a Varian-500 NMR spectrometer operating at a spectral frequency of 499.74 MHz at 298 K. One-dimensional (1-D) ^{1}H NMR spectra were obtained using a PRESAT pulse sequence to suppress the residual water resonance and 6,009 Hz spectral width with a 2.0 s relaxation delay. A total of 256 transients were collected into 16,384 data points requiring a ca. 20 min acquisition time. All data sets were zero filled to 32,768 data points and exponential line-broadenings of 0.5 Hz were applied before Fourier transformation. All ^{1}H NMR spectra were manually phased, baseline-corrected, and calibrated (DSS at 0.0 ppm) using Chenomx NMR Suite (version 5.1; Chenomx Inc., Edmonton, Canada) software. Peaks within the ^{1}H NMR spectra were assigned using the Chenomx 500-MHz library and public databases. Chenomx NMR Suite was also used for metabolite quantification (Cappello *et al.* 2017a, Cappello *et al.* 2017b, Maisano *et al.* 2017).

2.8 Statistical analysis

In order to test survival, hatch, length, deformity, and behavior, a General linear mixed model (GLM with Statistica software) was applied. When the results from GLM indicated a significant difference, a Newman-Keuls post hoc test was applied to compare groups. Significance levels were set at $p < 0.05$.

Statistical analyses for the metabolite data were conducted by the use of the GraphPad software (Prism 5.0, San Diego CA, USA). A one-way analysis of variance (ANOVA) was performed, followed by Dunnett's post-test, in order to determine the effects of single treatment groups compared to controls. Significance levels were set at $p < 0.05$.

3. RESULTS

Imidacloprid did not cause an impact on survival for any of the exposure conditions in either species of fish (data not shown). Moreover, overall hatching rate was unaffected for both

264 species despite a significant but transient alteration in hatching for some of the imidacloprid
265 concentrations at 7 dpf, and 8 dpf in medaka (SI Table S2). Yet, significant sub-lethal effects
266 were observed and these were more prominent for medaka as described below.
267
268 *3.1 Impact on fish development and behavior*
269
270 While no chemical-induced deformities and lesions were seen in zebrafish, the percentage of
271 total anomalies in medaka reached 67% at the lowest tested imidacloprid concentration, 0.2
272 µg/L, and >80% at 2 µg/L and higher (Figure 1A). Lordosis/scoliosis, hemorrhage and
273 jaw/skull deformity appeared in a concentration-dependent manner at ≥ 0.2 µg/L
274 imidacloprid; oedema of the yolk and bones as well as tail deformities became visible at
275 concentrations ≥ 20 µg/L (Figure 1B).
276
277 The stark difference in responding with sub-lethal effects between zebrafish and medaka
278 was also underlined in the histology. While no changes were found in the microscopic
279 structure of the eyes of zebrafish (Figure S3A, S3B), medaka larvae from 0.2 µg/L to the
280 highest concentration of imidacloprid exhibited a moderate disorganization of the retinal
281 pigment epithelium (Figure S3C, S3D). Additionally, a marked thickening of muscle fibers
282 was observed in zebrafish treated with 2000 µg/L of imidacloprid (Figure 2A, 2B) whereas in
283 medaka an altered myomeric structure, as highlighted by heterogeneous alignment of the
284 fibers and presence of white spaces among them, was evident starting from the exposure of
285 ≥ 2 µg/L of imidacloprid (Figure 2C, 2D).
286
287 Fish growth, measured as total length, was impacted by imidacloprid exposure in medaka
288 but not in zebrafish. All imidacloprid exposed medaka larvae were about 5% smaller than the
289 unexposed control group though this effect was not concentration-dependent (SI Table S3).
290

No impact by imidacloprid was found for both fish species for behavior. It pointed toward hypoactivity though without a clear concentration-dependent pattern (Figure 3).

3.2 Toxicokinetics of imidacloprid and its biotransformation products in the developing fish

Imidacloprid was taken up by the developing fish. Yet, as demonstrated especially for medaka with its longer development phase, the chorion provided a significant barrier for uptake – only between 7 to 10% of the final organism-internal concentration were detected in the organisms at this early life stage. Imidacloprid was below the detection limit in control whereas concentrations in the exposed larvae drastically increased immediately after hatch, reaching 60 to 80% of the concentration measured in the larvae at the end of exposure (Table 1 column 1 and 2 and SI Table S4).

Apparent bioconcentration factors (BCFs) were calculated at the end of the exposures by dividing the concentration of imidacloprid in the organism by the concentration measured in the water recognizing that steady-state conditions may not have been reached. These BCFs amounted to 1.5 L/kg$_{\text{wet weight (ww)}}$ and 1.2 L/kg$_{\text{ww}}$ for medaka and zebrafish, respectively. In contrast in medaka just before hatch, the BCF was 0.1 L/kg$_{\text{ww}}$.

Of the total imidacloprid quantified at the respective end of the exposures, i.e. in 5 dpf zebrafish and in 14 dpf medaka, about 15% were biotransformed as estimated based on the presence of three biotransformation products (Table 1 and SI Table S4). The predominant product in both species was hydroxyl-imidacloprid, which accounted for about 11% of biotransformation. Urea-imidacloprid was found at less than 1% in both species. Finally, olefin-imidacloprid represented 1% in zebrafish and about 3% in medaka. Desnitro-imidacloprid, which has been described in bees (Suchail *et al.* 2001), was not detected in either species.

3.3 Metabolic responses in medaka

To better understand the responses of medaka to imidacloprid exposure, we monitored the levels of endogenous metabolites in medaka larvae. Compared with larvae from controls (see SI Figure S4 for a representative 1-D ^{1}H NMR spectrum of 14 dpf medaka larvae), the exposure to imidacloprid resulted in significant changes ($p < 0.05$) in metabolites which can be assigned to either energy metabolism (namely glucose, pyruvate, succinate, ATP/ADP, and lactate) or to cholinergic (choline and acetylcholine) and to adrenergic (tyrosine and phenylalanine) neurotransmission (Table 2 and SI Figure S4).

4. DISCUSSION

The aim of our study was two-fold: (1) to explore if sub-lethal effects, ranging from impacts on development to behavior, can arise from imidacloprid early life stage exposure of two model fish – zebrafish and medaka – embracing concentrations close to environmental levels; and (2) to test if species differences arise.

Imidacloprid caused sublethal effects in both species but the effects were much more severe in medaka. The most prominent impact was the induction of deformities and lesions. The mechanisms leading to such effects can be manifold and will need further investigations to be precisely understood. However, several lines of evidence point toward an involvement of nicotinic acetylcholine receptors (nAChRs), i.e. the specific target of imidacloprid in insects, keeping in mind that, while in insects nAChRs are restricted to the central nervous system, these same receptors are also present at neuromuscular junctions in vertebrates (Millar *et al.* 2009).

The first line of evidence for an involvement of nAChRs is that the anomalies herein observed in the muscle structure of medaka larvae, with the heterogeneous alignment of the fibers, could be explained by a dysregulation of muscle contractions due to interference by

346 imidacloprid with the fish neuromuscular nAChRs. The exposure to imidacloprid can provoke
347 muscle contraction with consequent release of lactate, as evidenced by an increase in
348 lactate levels at the lowest exposure concentrations. Increased levels of acetylcholine
349 comprise the second line of evidence of the interference of imidacloprid with the fish
350 nAChRs. Indeed, increased acetylcholine levels recorded in all the exposure groups are in
351 agreement with the action and target-site selectivity of imidacloprid that saturates nAChRs
352 (Matsuda *et al.* 2009), leading to an increase of free acetylcholine. Similar effects can be
353 elicited in zebrafish, as demonstrated by Tufi et al (2016), but only at concentrations at least
354 one order of magnitude higher than the highest concentration tested here. Along the lines of
355 these sensitivity differences, the marked thickening of muscle fibers in zebrafish larvae at the
356 highest concentration of imidacloprid in our study was 2000-fold higher than the lowest
357 concentration at which this effect was observable in medaka. We therefore conclude that the
358 neuromuscular nAChRs might be an important target of imidacloprid especially during early
359 developmental stages in both species. The difference between medaka and zebrafish could
360 be due to a greater affinity of the medaka nAChRs to imidacloprid or an overall higher
361 activation of nAChRs due to greater time-integrated imidacloprid exposure levels in the
362 developing medaka. Moreover, besides the alterations in the cholinergic system,
363 disturbances in the adrenergic system were also detected herein in medaka, with increased
364 level of phenylalanine, a precursor of tyrosine, which was, however, not followed by an
365 increase in the level of tyrosine itself. Similar data were observed in zebrafish larvae after
366 exposure to imidacloprid, which induced increased levels of phenylalanine but no change in
367 the levels of tyrosine (Tufi *et al.* 2016).
368
369 Zebrafish and medaka are similar in many traits, such as size, optimal temperature range
370 and being oviparous (Furutani-Seiki *et al.* 2004). However, the developmental time from
371 fertilization to free swimming larvae is about three times longer in medaka than in zebrafish
372 (14 d for medaka, 5 d for zebrafish) (Kimmel *et al.* 1995, Furutani-Seiki *et al.* 2004, Iwamatsu
373 2004)

At time of hatching (9 d for medaka, 3 d for zebrafish), medaka larvae contained about twice the amount of imidacloprid per g of tissue compared to zebrafish larvae. If passive uptake into the embryo is assumed, the higher accumulation in medaka larvae could be explained by the longer exposure time, assuming that steady-state-concentrations have not been reached. Yet another factor influencing accumulation could be the difference the composition of the chorion between medaka and zebrafish. While both zebrafish and medaka have a transparent chorion with 3 layers (Bonsignorio *et al.* 1996), there are differences in certain traits. The chorion of zebrafish is soft compared to the chorion of medaka and is as well smooth compared to the hair structures on the medaka chorion surface. The relative hardness of the medaka chorion could be due to a higher content of proline (Bonsignorio *et al.* 1996), which is known to contributes to the structural stability of proteins like collagen (Jaeken 2012). Such features, i.e. biochemical compositions and surface structures like hair, could contribute to the medaka chorion being more prone to chemical uptake than the chorion of zebrafish.

After hatch, imidacloprid concentrations rose in both species of fish though overall apparent BCFs indicate that imidacloprid does not accumulate strongly in both species (1.5 $L/kg_{wet\ weight\ (ww)}$ and 1.2 L/kg_{ww} for medaka and zebrafish, respectively). This is in accordance with BCFs for imidacloprid reported for other species of fish (*australoheros facetus* (Iturburu *et al.* 2016) (1.4L L/kg_{ww})) or amphibians (Van Meter *et al.* 2016) (0.2 to 0.7L L/kg_{ww}) whereas in gammarids, the BCF was higher (7.35 L/kg_{ww}) (Ashauer *et al.* 2010, Ashauer *et al.* 2012). At the end of the exposure, medaka showed a 1.3-fold higher accumulation compared to zebrafish, which contrasts with the orders of magnitude difference in developmental sensitivity of the medaka compared to zebrafish. Thus, while longer exposure time, paired with greater internal exposure, are conceivable contributors to the comparatively high sensitivity of medaka, other factors appear to contribute to the species sensitivity differences. One such factor could be biotransformation though the only notable difference was in the formation of olefin-imidacloprid. Interestingly, olefin-imidacloprid was found to be twice as lethally toxic than the parent compound in bees after 48 hours of exposure (Suchail *et al.*

2001). It was also shown to be potentially more toxic in mice (Lee Chao *et al.* 1997, Tomizawa *et al.* 1999). Thus, exploration of sensitivity toward olefin-imidacloprid is one future avenue to shed light on the species differences observed.

The comparatively high sensitivity of medaka toward imidacloprid during early development underlines the importance of taking species differences for environmental risk assessment into account. As demonstrated in the species sensitivity distribution (SI Figure S1), only few species of fish have thus far been explored for their sensitivity to imidacloprid with a focus on acute exposure. Though the exact mechanisms of the high sensitivity of the medaka during early life stages still need to be further explored, this fish appears about three orders of magnitude more sensitive to imidacloprid than the zebrafish. The most important impacts measured herein with regard to ecological relevance are the developmental toxicity and the reduced growth of medaka. Both these types of impact can conceivably be linked. For example, the alteration of muscle fibers can result in reduced locomotion, which in turn can result in reduced ability to catch food. This thought is supported by the observed tendency toward hypoactive behavior and disturbances in the neurotransmission pathways, both in the cholinergic and adrenergic systems. Similarly, alterations to the structure of the eyes may obstruct perception of predator or prey. All these impacts therefore can severely hamper the fitness of the fish in their natural environment. In the future, research priorities to further explore the species sensitivity differences could be to *i*) explore the barrier function of the chorion on chemical uptake, *ii*) mechanisms of neuromuscular nAChRs and *iii*) the toxicity of olefin-imidacloprid in both species. In this regard it is important to note that 0.2 µg/L of imidacloprid, i.e. the lowest concentration at which strong effects were already seen in medaka, is in the range of concentrations (µg/L) reported in some environments like rivers, groundwaters, streams and estuaries (Anderson *et al.* 2015, Morrissey *et al.* 2015).

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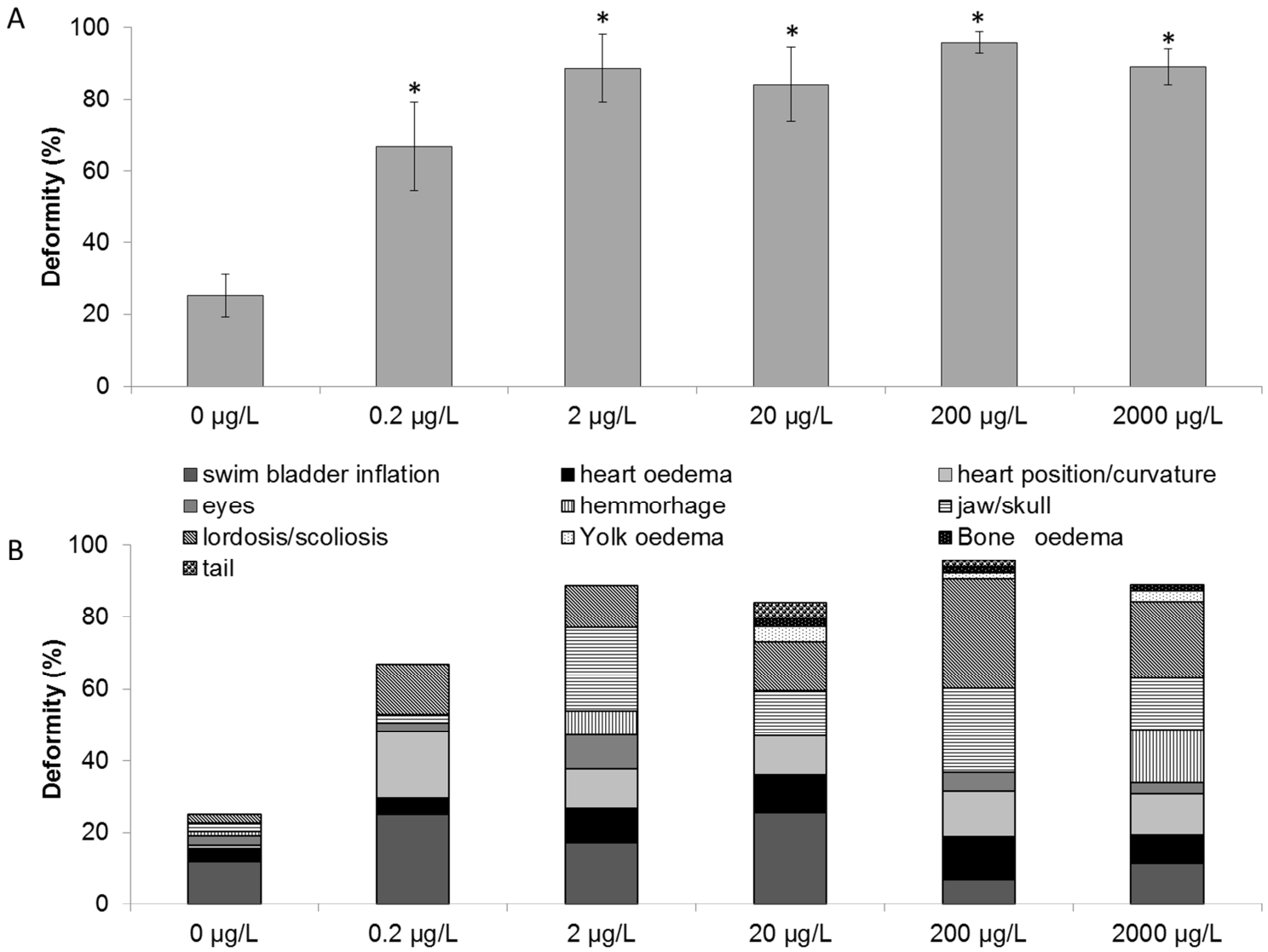
6. Declarations of interest

None.


A
Deformity (%)
100
80
60
40
20
0
*
*
*
*
0 µg/L
0.2 µg/L
2 µg/L
20 µg/L
200 µg/L
2000 µg/L
swim bladder inflation
heart oedema
heart position/curvature
eyes
hemmorhage
jaw/skull
lordosis/scoliosis
Yolk oedema
Bone oedema
tail
B
Deformity (%)
100
80
60
40
20
0
0 µg/L
0.2 µg/L
2 µg/L
20 µg/L
200 µg/L
2000 µg/L

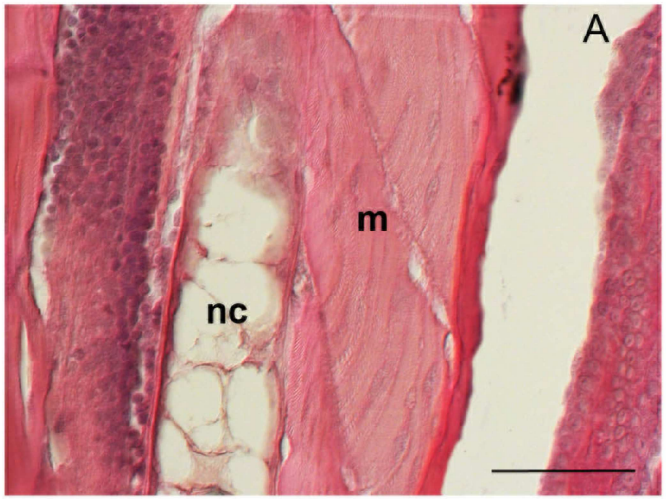
441

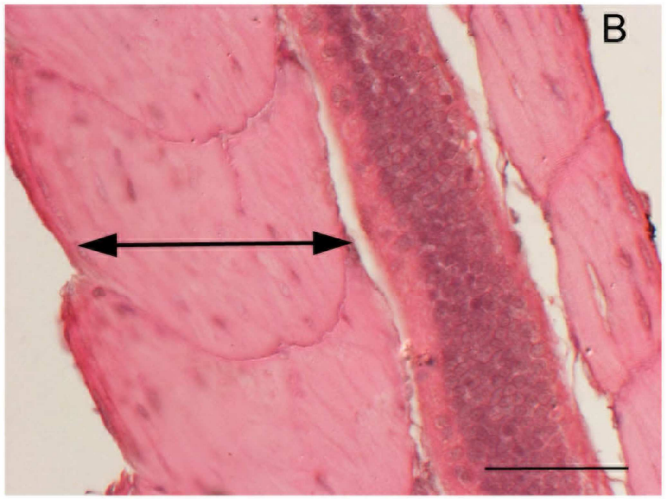
Figure 1. Deformities in medaka at hatch (9 dph). A. Percentage of total developmental anomalies. Stars indicate significant differences to control fish (0µg/L) for $p \leq 0.05$ as determined by GLM. B. Anomalies are ranked into three categories. Group 1: non chemical-specific anomalies (plain grey to dark); they include lack of swim bladder inflation, ocular lesion (missing eye, cyclopia and dystrophy), heart oedema and heart position/curvature. Group 2: concentration dependent anomalies (dashed line patterns); they include lordosis/scoliosis, hemorrhage and jaw/skull deformity. Group 3: anomalies that became visible only at concentrations ≥ 20 µg/L (dotted patterns); they include oedema of the yolk and bones as well as tail deformity. These experiments was done 3 times with 10 embryos per treatment each time.

Control

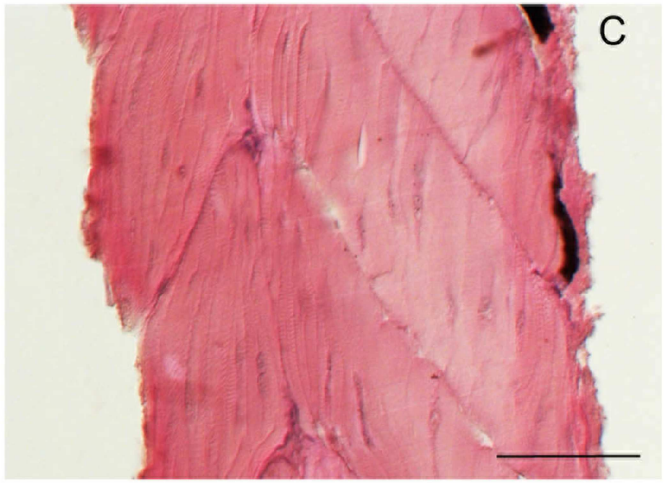
Imidacloprid

Zebrafish





Medaka



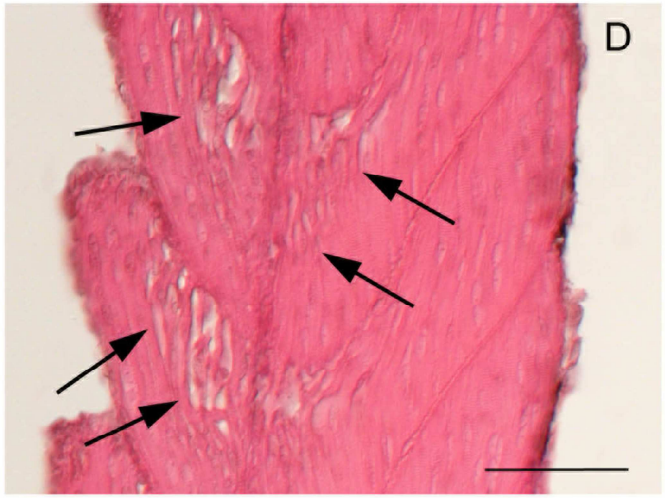


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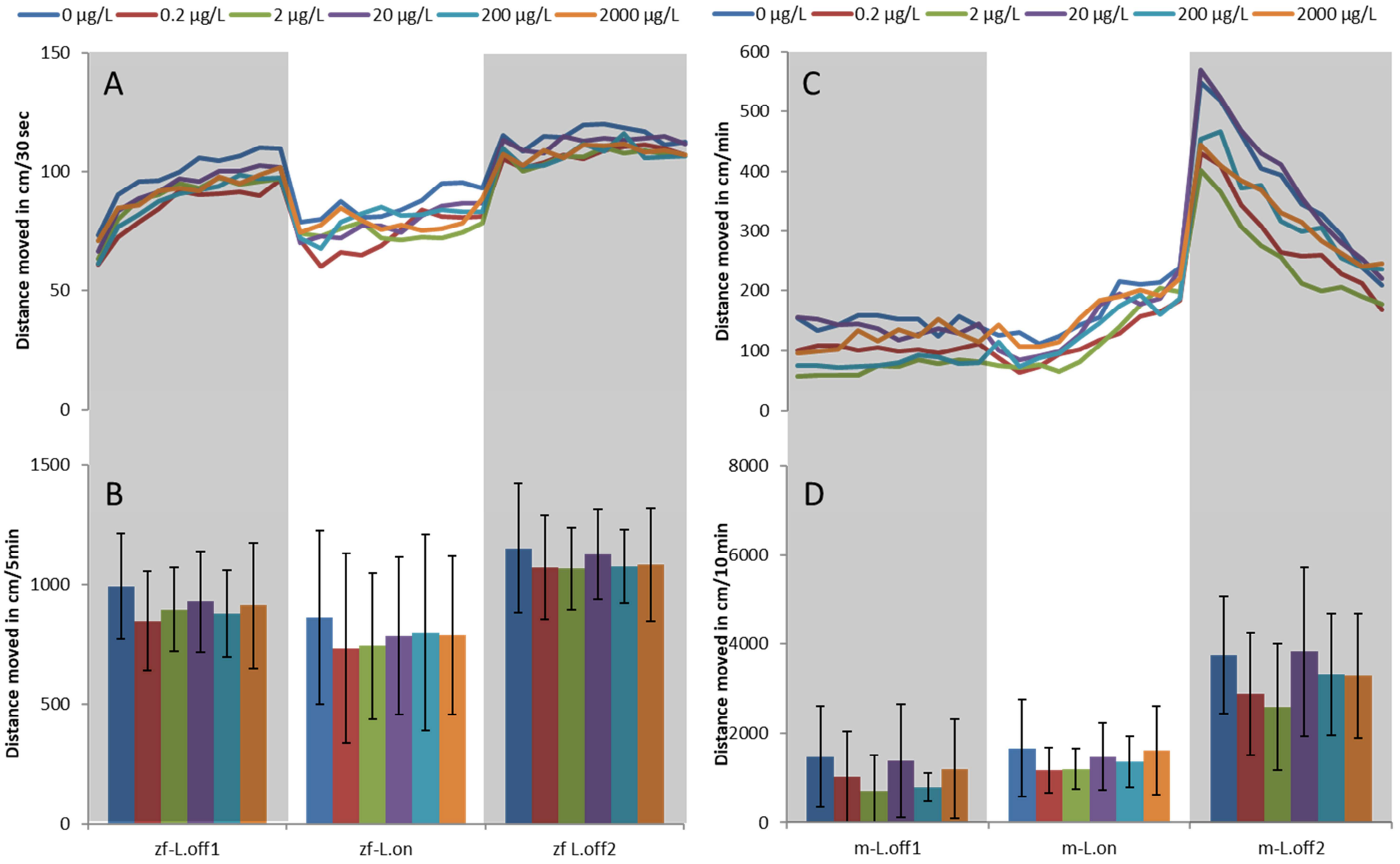
Figure 2. Histological sections of muscles of 5 dpf zebrafish (A-B) and 14 dpf medaka (C-D), stained with Hematoxylin and Eosin. A regular microscopic structure of the muscles of zebrafish from control group (A) and marked thickening of muscle fibers (double ended arrow) in zebrafish treated with 2000 µg/L of imidacloprid (B). Muscles of medaka from control (C) and a representative image showing an altered myomeric structure with heterogeneous alignment of the fibers (arrows), and presence of white spaces among them, found from 2 µg/L to the highest concentration of imidacloprid group (D). nc, nothocord; m, muscle. Scale bars, 20 µm.



Zebrafish

Medaka

466

Figure 3. Behavioral response in zebrafish after 5 days of exposure (A, B) and in medaka after 14 days of exposure (C, D) to different concentrations of imidacloprid. (A). Distance moved measured every 30 sec. (B). Distance moved measured every 5 minutes. (C). Distance moved measured every minute. (D). Distance moved measured every 10 minutes. Zf-Loff1: zebrafish Light off1 (5 min); Zf Lon: zebrafish Light On (5 min); Zf-Loff2: zebrafish light off 2 (5 min); m-Loff1: medaka Light off1 (10 min); m-Lon: medaka Light On (10 min); m-Loff2: medaka light off 2 (10 min).

Table 1: Whole body internal concentration of imidacloprid and biotransformation products in ng/g w.t after imidacloprid exposure (n.d= not detected; n.q= detected but under limit of quantification (see Table S4).

		Imidacloprid	Hydroxyl-imidacloprid	Desnitro-imidacloprid	Olefin-imidacloprid	Urea-imidacloprid
zebrafish	Unexposed larvae (3, 5 dpf)	n.d	n.d	n.d	n.d	n.d
	Larvae at hatching day (3 dpf)	1267 ± 58	64 ± 4	n.q	9 ± 1	8 ± 0.5
	Larvae at the end of experiment (5 dpf)	2067 ± 153	263 ± 31	n.q	22 ± 3	11 ± 1
medaka	Unexposed embryo (3, 5 dpf) and larvae (9,14 dpf)	n.d	n.d	n.d	n.d	n.d
	3 dpf (embryo)	180 ± 35	n.d	n.d	n.d	n.d
	5 dpf (embryo)	273 ± 12	n.d	n.d	n.d	n.q
	Larvae at hatching day (9 dpf)	2133 ± 115	160 ± 20	n.d	24 ± 3	11 ± 1
	Larvae at the end of experiment (14 dpf)	2667 ± 252	390 ± 36	n.d	76 ± 8	12 ± 1

477 **Table 2.** Percent changes in concentrations of metabolites between imidacloprid-exposed and control medaka (Dunnett's test; *$p < 0.05$).

	0.2 µg/L	2 µg/L	20 µg/L	200 µg/L	2000 µg/L
Metabolites relative to energy metabolism					
Glucose	↓ 10%	↓ 13%	↓ 24%	↓ 38%	↓ 31%
Pyruvate	↑ 64%*	↑ 70%*	↑ 70%*	↑ 39%	↑ 43%
Succinate	↑ 62%*	↑ 75%*	↑ 58%*	↑ 14%	↑ 4%
ATP/ADP	↑ 30%	↑ 42%*	↑ 40%*	↓ 14%	no change
Lactate	↑ 52%*	↑ 47%*	↑ 50%*	↓ 46%*	↓ 21%
Metabolites relative to cholinergic neurotransmission					
Choline	↑ 24%	↑ 40%*	↑ 16%	↓ 23%	↓ 12%
Acetylcholine	↑ 64%*	↑ 44%*	↑ 34%	↑ 25%	↑ 17%
Metabolites relative to adrenergic neurotransmission					
Tyrosine	↑ 66%*	↑ 29%	↑ 16%	↓ 10%	no change
Phenylalanine	↑ 78%*	↑ 70%*	↑ 104%*	↑ 68%*	↑ 37%

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615

HIGHLIGHTS:

- Imidacloprid impacts on fish at environmentally relevant concentrations.
- At the same developmental stage, medaka are more sensitive than zebrafish.
- Our study supports the importance of taking species sensitivity differences into account