Different developmental insecticide exposure windows trigger distinct locomotor phenotypes in the early life stages of zebrafish

Melissa von Wyl [a,1], Sarah Könemann [a,b,1], Colette vom Berg [a,*]

[a] Department of Environmental Toxicology, Eawag, Überlandstrasse 133, 8600 Dübendorf, Switzerland
[b] École Polytechnique Fédéral de Lausanne, EPFL, Route Cantonale, 1015 Lausanne, Switzerland

HIGHLIGHTS

- Tail coiling, touch-evoked response, and locomotion were differently affected.
- The outcome of a later behavioral endpoint cannot be inferred from an earlier phenotype.
- Previously observed behavioral effects recovered in absence of the insecticides.
- No particularly critical developmental window was identified.

GRAPHICAL ABSTRACT
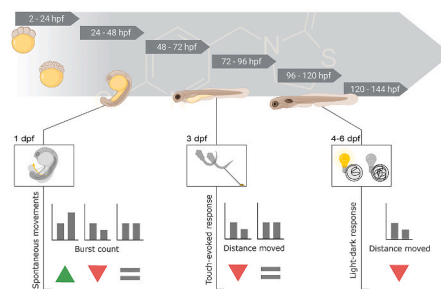
ARTICLE INFO

ABSTRACT

Due to their extensive use and high biological activity, insecticides largely contribute to loss of biodiversity and environmental pollution. The regulation of insecticides by authorities is mainly focused on lethal concentrations. However, sub-lethal effects such as alterations in behavior and neurodevelopment can significantly affect the fitness of individual fish and their population dynamics and therefore deserve consideration. Moreover, it is important to understand the impact of exposure timing during development, about which there is currently a lack of relevant knowledge. Here, we investigated whether there are periods during neurodevelopment of fish, which are particularly vulnerable to insecticide exposure. Therefore, we exposed zebrafish embryos to six different insecticides with cholinergic mode of action for 24 h during different periods of neurodevelopment and measured locomotor output using an age-matched behavior assay. We used the organophosphates diazinon and dimethoate, the carbamates pirimicarb and methomyl as well as the neonicotinoids thiacloprid and imidacloprid because they are abundant in the environment and cholinergic signaling plays a major role during key processes of neurodevelopment. We found that early embryonic motor behaviors, as measured by spontaneous tail coiling, increased upon exposure to most insecticides, while later movements, measured through touch-evoked response and a light-dark transition assay, rather decreased for the same insecticides and exposure duration. Moreover, the observed effects were more pronounced when exposure windows were temporally closer to the performing of the respective behavioral assay. However, the measured behavioral effects recovered after a short period, indicating that none of the exposure windows chosen here are particularly critical, but rather that insecticides acutely interfere with neuronal function at all stages as long as they are present. Overall, our results contribute to a better

* Corresponding author.
  E-mail address: Colette.vomberg@eawag.ch (C. vom Berg).
[1] The authors contributed equally to the preparation of the manuscript.

understanding of risks posed by cholinergic insecticides to fish and provide an important basis for the development of safe regulations to improve environmental health.

1. Introduction

Chemical pollution and waste is a major threat to human and environmental health and has recently received more attention on the agendas of policy bodies (Bernhardt et al., 2017; Brack et al., 2022; Groh et al., 2022). For most chemicals in use, substantial knowledge gaps exist with regard to their human and environmental health hazard, which prevents their sound management and the development of safe environmental quality standards. One group of regulatable chemicals, for which a more refined assessment of hazard can potentially lead to significantly increased environmental safety, are insecticides. They are extensively used all over the world to control pests and pathogens in medicine, households and agriculture. Via spray drift, leaching, run-off and wastewater discharge they reach the aquatic environment (Schulz, 2004), where they pose a risk to non-target organisms such as fish. To protect the environment and develop safe environmental quality standards, the hazards posed by insecticides to fish need to be assessed. However, most insecticides have so far only been thoroughly assessed for lethality, while more subtle effects such as behavioral alterations and neurodevelopmental toxicity, which can severely reduce an organism's chances of survival, are more difficult to grasp and interpret despite the considerable number of studies available. Therefore, behavior- and neurotoxicity-related endpoints have thus far not been considered in environmental protection (Ford et al., 2021). Moreover, some insecticides, such as imidacloprid and thiacloprid, have been identified as contaminants of emerging concern for which more monitoring data is needed to comprehensively assess the risk for the aquatic environment. Therefore, these chemicals, among others, have been included in the EU Watch list, indicating persistent concern associated with them in Europe and worldwide.

In continental Europe and other regions of the world, the insecticide spraying season often coincides with the spawning season of many fish species. For example, a monitoring study found pesticides in the range of 1 ng/L to 10 μg/L in small streams from mid-March to August, with the number of detected substances ranging from 69 to 98 in five catchments (Spycher et al., 2018). Likewise, the rise in temperature and photoperiod are key environmental factors that drive reproduction in spring-spawning fish species (Arevalo et al., 2020). Thus, these fish species are exposed to a high number of pesticides during the early life stages of their development.

Zebrafish (*Danio rerio*) are tropical freshwater fish abundant in rice paddies and slow-moving streams in India and Burma. They are frequently used as model species in toxicology and other fields of research because they are easy to maintain and regularly produce large numbers of small, transparent and rapidly developing embryos, which are considered non-protected animal stages up to 5 days of age. These features enable high-throughput testing and the assessment of effects from fertilization onwards without compromising maternal influence. Zebrafish locomotor assays are widely applied to assess the impact of neuroactive chemicals on the nervous system (Fitzgerald et al., 2021) because locomotor behavior reflects the combined action of neuronal, neuroendocrine and neuromuscular signals and is readily measurable in zebrafish early life stages. Importantly, the ontogeny of zebrafish locomotor behavior and the underlying maturation of the locomotor network has been subject to extensive studies and is well characterized (Drapeau et al., 2002; Brustein et al., 2003). The first embryonic movements start around 17 h after fertilization and are characterized by spontaneous coiling contractions of the trunk (Kimmel et al., 1995). A few hours later, the embryo becomes sensitive to touch and initially responds to it with vigorous coiling, and later on with an escaping swim burst. After hatching, within 2–3 days, the larvae swim spontaneously,

although infrequently and in bursts, which slowly transitions into a beat-and-glide swimming mode after swim bladder inflation and right before the feeding stage at 5 days (Saint-Amant and Drapeau, 1998, Drapeau et al., 2002). The appearance of these motor behaviors is governed by cellular changes in the underlying developing locomotor network (Buss and Drapeau, 2001; Saint-Amant and Drapeau, 2001, Brustein et al., 2003). Due to these well described processes and the wide range of tools available, the zebrafish model offers the opportunity to gain mechanistic insights in chemical-induced behavioral changes. For these reasons, it has been used in the present study, although of limited ecological relevance due the small region in Asia where it is found in the wild.

The developing nervous system of any species is particularly prone to perturbations because it involves complex processes, which are temporally and regionally highly coordinated. These processes involve proliferation, migration, differentiation, synaptogenesis, myelination and apoptosis, and are largely conserved between mammals and other vertebrates such as fish (Rice and Barone, 2000; Schmidt et al., 2013). Neuroactive insecticides have the potential to interfere with these processes, thereby preventing the development of a properly functioning nervous system (Barone et al., 2000). Disturbance of these different cellular processes can lead to changes in locomotor behavior, with some processes being more susceptible than others and thus constituting particularly vulnerable periods. In toxicology, chemical exposure during such sensitive periods in development, also referred to as critical windows, leads to maladaptive phenotypes while exposure during other periods may not be as severe (Burggren and Mueller, 2015). One group of chemicals for which critical windows are well described are endocrine disrupting chemicals. They have been shown to affect sexual differentiation and gonadal development among several fish species during a specific window of time in early larval development when the reproductive system is particularly plastic (Koger et al., 2000; van Aerle et al., 2002; Ankley and Johnson, 2004; Maack and Segner, 2004). Studies on the neurotoxic effects of insecticides in fish have mostly focused on continuous exposure during development and analyzed either early embryonic behavior, such as spontaneous tail coiling, or effects on beat-and-glide swimming performance e.g. during light-dark transition tests. Exposure of zebrafish embryos to organophosphates triggered hypoactivity in early embryonic movements and larval locomotion in most studies (Kienle et al., 2009; Selderslaghs et al., 2010; Yen et al., 2011; Watson et al., 2014, Jarema et al., 2015; Cao et al., 2018; Schmitt et al., 2019), with a few exceptions where hyperactivity was measured (Selderslaghs et al., 2010; Leuthold et al., 2019). Similarly, continuous exposure of zebrafish embryos to neonicotinoids and GABA receptor blockers affected spontaneous coiling and locomotor activity at later stages (Stehr et al., 2006; Von Hellfeld et al., 2022). However, sensitive windows for insecticide exposures in fish have thus far only scarcely been assessed, despite their potential to interfere with temporally restricted cellular processes during neurodevelopment. Furthermore, how well early insecticide-induced locomotor phenotypes correspond to later locomotor effects for the same chemicals and for the same exposure duration has not been thoroughly investigated.

Therefore, the aim of the present study was to close this knowledge gap by investigating whether there are periods during the neurodevelopment of fish that are particularly vulnerable to insecticide exposure. To address this question, we exposed zebrafish embryos to insecticides for 24 h during different periods of neurodevelopment and measured locomotor output using an age-matched assay (spontaneous tail coiling, touch-evoked response, light/dark transition test). We used the following cholinergic insecticides: the organophosphates diazinon and dimethoate and the carbamates pirimicarb and methomyl, all of

which inhibit acetylcholine esterase, as well as the neonicotinoids imidacloprid and thiacloprid, which bind to nicotinic acetylcholine receptors because 1) they are widely used and measured frequently in the aquatic environment of agricultural areas and 2) the cholinergic machinery plays a major role during the nervous system development of fish (Rima et al., 2020). This study is based on previous observations showing that the selected insecticides reduced locomotor behavior and induced developmental neuromuscular alterations (Könemann et al., 2022). As a follow-up, we further explored critical windows of exposure to the previously tested concentrations of these insecticides.

2. Materials and methods

2.1. Fish maintenance

Zebrafish (*Danio rerio*) of the AB line were maintained as described by Fitzgerald et al. (2019). The Cantonal Veterinary Office Zurich (CH) approved all experiments involving larvae under the license ZH168/17 and ZH134/2021.

2.2. Insecticide exposure

Analytical grade (PESTANAL, ≥98% purity) insecticides were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, DE). The stock solution was freshly prepared for each experiment by dissolving the test substance in embryo medium (294.0 mg/L $CaCl_2 \times 2H_2O$, 123.3 mg/L $MgSO_4 \times 7H_2O$, 64.7 mg/L $NaHCO_3$, and 5.7 mg/L KCl in nanopure water) prepared according to ISO (International Organization for Standardization) guidelines (Standardization, 1999). Prior to the experiment, the stock was diluted with embryo medium to prepare the final test concentration (nominal) of 4.68 mg/L (diazinon), 150 mg/L (dimethoate), 2.89 mg/L (methomyl), 30.55 mg/L (pirimicarb), 150 mg/L (imidacloprid) and 125 mg/L (thiacloprid). These concentrations were selected based on a previous study, which investigated the effects of the same insecticides on locomotion and the structural development of the neuromuscular synapse (Könemann et al., 2022). As a first step, the fish embryo toxicity test (96 h) was used to determine the non-toxic concentration (NtC) for each insecticide. The effects of NtC and 1.5xNtC on locomotion were then tested in the light-dark transition test and the development of neuromuscular structures was investigated using immunofluorescence (results summarized in SI, Table S1). For almost all tested insecticides used in the previous study, both concentrations led to

a reduction in locomotion, while in some cases only the higher concentration (1.5xNtC) induced structural effects. Therefore, in the present study, we focused on this worst-case scenario and followed up on the previous findings by determining whether there is a critical window of exposure. Therefore, 18 zebrafish per treatment were exposed during separate 24-h windows from 2 to 144 hpf (Fig. 1). To allow for batch assessment of spontaneous tail coiling, embryos were kept in petri dishes from 2 to 24 hpf (see section 2.4). Afterwards, all embryos were transferred to three 48-well plates (from 24 until 144 hpf) for touch response and locomotion assessment (see section 2.5 and 2.6). From that time point on, larvae were transferred to the respective exposure substance during the exposure windows and then back into clean embryo medium, always in the 48 well plates. Control larvae and exposed larvae underwent the same transfer at 48 hpf from the petri dishes to the 48-well plates. Continuously exposed larvae remained in the exposure medium also during the behavioral assessments. Due to the high adsorption potential of diazinon to the plastic, the continuous exposure of diazinon was tested semi-statically (medium exchange every 24 h) while the other insecticides were tested statically. We have considered individual embryos as independent replicates.

2.3. Chemical analysis

Final exposure concentrations were assessed using liquid chromatography and mass spectrometry (LC - MS). Samples were taken at the beginning and end of the exposure and processed, as previously described (Könemann et al., 2021). Briefly, samples were separated using liquid chromatography on a C18 Nucleodur column and subsequently measured on a quadrupole orbitrap mass spectrometer (Q Exactive Plus, Thermo Fisher Scientific Inc., USA) with electrospray ionization in positive ion mode. Detailed information can be found in the supplementary information page 2.

2.4. Spontaneous tail coiling test

Spontaneous tail coiling of 24 hpf embryos was assessed as previously described by Jin et al. (2009). Per concentration, a batch of 18 embryos in a petri dish was video tracked for 60 s using a Basler acA 2000-165 µm camera mounted on a Leica S8APO stereo microscope and the Media Recorder 4 software. Afterwards, the number of spontaneous tail coils (shown as tail coils/minute) as well as the duration of tail coils in seconds was analyzed using the DanioScope software (Noldus, NL). In
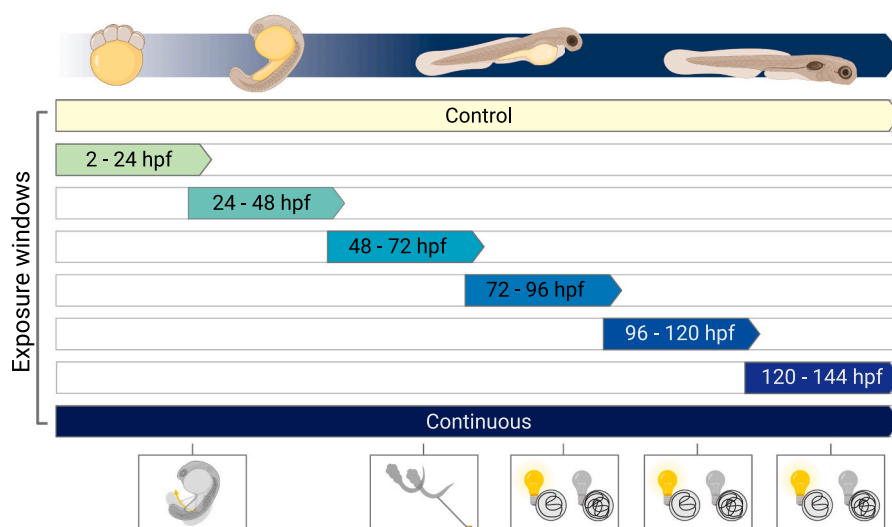
Fig. 1. Overview of the exposure scheme and the assays conducted at different points in the larvaes' development. Embryos/Larvae were exposed during one of the shown 24-h exposure windows and were then subjected to the spontaneous tail coiling test (at 24 h), the touch-evoked response test (at 72 h), and the light-dark response test (at 96, 120, and 144 h).

order to allow the embryos to acclimate and calm down after being moved from the incubator to the microscope, 2 min of acclimation time were added prior to recording. The light condition under which the assay was conducted was 787 lux (or 11 PFD).

2.5. Touch-evoked response test

After hatching at approximately 72 hpf, the touch-evoked response (TER) test was conducted based on a protocol previously described by Guzman et al. (2020). Individual recordings of single larvae per well were taken in a 48-well plate. Each larva was filmed and recorded using the same equipment and software as for the spontaneous tail coiling assay, as well as the same light conditions. After 10 s, the tail was touched with a stainless steel wire (Ø 0.10 mm). This was repeated after 20 s and 30 s. The total length of the video was 40 s. The recorded videos were quantitatively analyzed, as described by Guzman et al. (2020). Briefly, based on the rounds swum after the stimulus, larvae were grouped into different categories, such as zero to quarter rounds, quarter to half rounds, etc. (SI, Table S4). Using the circumference of the well of 32.67 mm, the rounds swum were converted to the distance moved.

2.6. Light-dark response test

In order to assess the locomotion of larvae at 4, 5, and 6 dpf, the light-dark transition (LDT) assay was carried out based on a previously described protocol (Fitzgerald et al., 2019). Videos were recorded with the DanioVision Observation Chamber (Noldus, NL) running on Etho-Vision XT (version 13). Larvae were first acclimated for 20 min in light followed by a spontaneous swimming tracking period of 20 min and two alternating dark and light periods of 10 min each (dark-light-dark-light). For the statistics, the total distance summed across both dark or both light periods was analyzed. The whole protocol has a duration of 80 min and started at 8.30 a.m. for the first plate and subsequently at 10 a.m. and 11.30 a.m. for the second and third well - plate. Each plate contained the negative control, continuous exposure, and all exposure windows (SI, Table S5). All locomotion experiments were conducted in a temperature-controlled room at 26 ± 1 °C and the light intensity amounted to 2412 lux (or 34 PFD) in the light periods.

### 2.7. Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 9.0). All data was tested for normality and log normality with the Shapiro-Wilk test (alpha = 0.05). If data was normally distributed, the unpaired t-test was applied to data with two groups (spontaneous tail coiling); otherwise the Mann-Whitney test was applied. A Brown-Forsythe and Welch ANOVA with Dunnett's multiple comparisons test was applied to normally distributed data with non-homogenous variances of data with multiple groups (TER assay, light-dark transition assay). If data was not normally distributed, the Kruskal-Wallis test with Dunn's multiple comparisons test was applied. A statistical significance threshold of $p < 0.05$ was used.

3. Results

No exposure-induced lethality, developmental delay or effects on hatching were observed for the following measured concentrations tested in this study: 4.27 mg/L diazinon, 146.74 mg/L dimethoate, 1.94 mg/L methomyl, 34.16 mg/L pirimicarb, 153.87 mg/L imidacloprid, and 138.51 mg/L thiacloprid (SI, Table S2 and S3). Further, larvae with dysmorphologies were removed and not used for the experiments.

3.1. Insecticides affect number and duration of tail coils

As shown in Fig. 2, we measured an increased mean number of tail coils/min after 24 h of exposure to dimethoate, methomyl, and pirimicarb. The strongest increase in tail coils/min was induced by treatment with pirimicarb, where the mean tail coils per minute increased by 261% (mean ± SD, 26.0 ± 14.2 tail coils/min, $p \leq 0.001$) compared to mean of the control group (7.2 ± 2.3 tail coils/min). Dimethoate-treated larvae showed an increase in the mean tail coils per minute of 38% (12.9 ± 7.2 tail coils/min, $p \leq 0.033$) and methomyl of 19% (9.3 ± 3.2 tail coils/min, $p \leq 0.033$) compared to the control groups (control$_{dimethoate}$ = 9.3 ± 4.5 tail coils/min, control$_{methomyl}$ = 7.8 ± 1.9 tail coils/min). Thiacloprid was the only insecticide that reduced the number of mean tail coils per minute (by 27% - 6.4 ± 2.2 tail coils/min, $p \leq 0.002$) compared to the control group (8.8 ± 2.6 tail coils/min). We observed that control larvae exhibited side-to-side flexes of the tail as previously
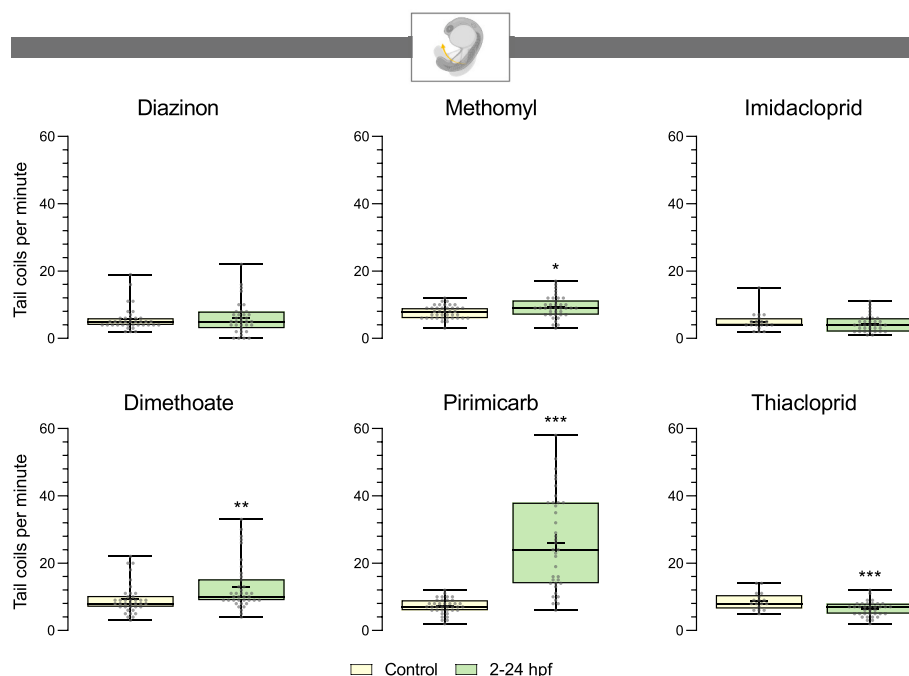
Fig. 2. Number of spontaneous tail coils expressed as tail coils per min after insecticide exposure from 2 to 24 h post fertilization. The boxplots show the mean (cross), median (line) and minimum and maximum values as whiskers. Each dot represents one fish. The Mann-Whitney or unpaired t-test was used to evaluate the statistical difference between the control treatment and the exposed larvae. The p values of the post-hoc test are * = $p \leq 0.033$, ** = $p \leq 0.002$, and *** = $p \leq 0.001$. The exact p values calculated by GraphPad Prism are shown in SI, Table S6.

described (Saint-Amant and Drapeau, 1998, Richendrfer et al., 2014), whereas exposed larvae sometimes lacked this side-to-side alternation so that the tail predominantly coiled in one direction. Otherwise, the tail coils of exposed larvae did not differ in strength or shape compared to those performed by control larvae. Larvae exposed to diazinon and imidacloprid did not alter in tail coiling activity compared to the control group. In addition to the number of the spontaneous tail coils, we analyzed the duration of tail coils. Thereby, diazinon-treated larvae showed a reduced duration of tail coils of 41% (mean ± SD, 0.30 ± 0.09 s, $p \leq 0.001$) and methomyl-treated larvae a reduction of 8% (0.31 ± 0.09 s, $p \leq 0.033$) compared to the control groups ($control_{diazinon} = 0.52 \pm 0.14$ s; $control_{methomyl} = 0.33 \pm 0.07$ s). All other Insecticides had no impact on the duration of tail coiling (see Fig. S1 in SI).

3.2. Insecticides can reduce TER response during exposure

To assess the locomotor performance during short burst movements of 72 hpf-old larvae, we conducted the TER assay. We observed a reduction in distance moved induced by the touch to the tail of zebrafish larvae exposed during 48–72 hpf for all insecticide-treatments except imidacloprid, as can be seen in Fig. 3. The reduction was most drastic for treatments with methomyl, which showed a reduction of 100% ($p \leq 0.001$), followed by thiacloprid with a reduction in mean distance moved of 76% (mean ± SD, 8.5 ± 7.9 mm, $p \leq 0.001$), dimethoate of 46% (18.4 ± 8.9 mm, $p \leq 0.033$) and diazinon of 44% (18.5 ± 8.3 mm, $p \leq 0.002$). Continuously exposed larvae to all insecticides except diazinon and imidacloprid showed a reduction in touch-evoked response, whereby methomyl exposed larvae were most affected, with a reduction of 98% (0.91 ± 2.29 mm, $p \leq 0.001$), and pirimicarb the least, with a reduction of 30% (27.53 ± 17.06 mm, $p = 0.05$) compared to the respective control group. The other insecticides led to the following reductions compared to the control group: pirimicarb: 30% (27.53 ± 17.06 mm, $p = 0.05$), dimethoate: 49% (17.62 ± 12.22 mm, $p \leq 0.001$), thiacloprid: 80% (6.89 ± 7.40 mm, $p \leq 0.001$), methomyl: 98% (0.91 ± 2.29 mm, $p \leq 0.001$). In addition, larvae exposed to pirimicarb from 24 to 48 hpf showed a reduction in the mean distance moved of 33% (26.4 ± 15.5 mm, $p = 0.05$). The control groups ranged

between a mean distance moved of 32.9–39.7 mm.

### 3.3. Reduced locomotion in the light-dark transition test

As shown in Fig. 4, all insecticides induced a reduction in the distance moved in the light-dark transition assay at a certain point during exposure. Continuously exposed larvae all showed a significant reduction in distance moved in the dark of 19–64%, except continuously treated larvae with diazinon at day 5 compared to the control group (day 4: 8.6–10.9 cm; day 5: 11.6–13.2 cm, day 6: 10.4–12.1 cm). The larvae exposed during the test (72–96 hpf for day 4, 96 to 120 hpf for day 5, 120 to 144 hpf for day 6) reduced their locomotion by 20–58% for all insecticide treatments, except imidacloprid at day 6, compared to the control group.

We could further observe a decrease in distance moved in dark periods, for larvae treated with diazinon. We observed a reduction of 22% at day 4, when exposed from 2 to 24 hpf as well as a reduction of 26% when exposed from 48 to 72 hpf compared to the control group. At day 5 as well as day 6, diazinon-treated larvae from 72 to 96 hpf reduced their movement by 25% and 18% respectively compared to the control group. Moreover, when investigating the behavior at day 6, larvae exposed to diazinon from 96 to 120 hpf showed a reduction in movement of 17% (mean ± SD, 19.55 ± 1.68 cm, 9.93 ± 2.26 cm) compared to the control group in the dark (11.49 ± 2.33 cm and 11.90 ± 1.64 cm). The same reduction in movement at day 6 could be observed, when larvae were exposed to dimethoate from 96 to 120 hpf, in the dark periods. Larvae treated with dimethoate or thiacloprid from 48 to 72 hpf displayed a reduction in locomotion at day 4 of 21% (dimethoate) and 30% (thiacloprid) compared to the control group. In contrast to these findings, larvae treated with methomyl from 48 to 72 hpf increased their movement by 30% (13.75 ± 3.78 cm) in the dark periods at day 6 compared to the control group (10.58 ± 2.15 cm).

During the light periods (see SI, Fig. S2), larvae generally moved less, a pattern well described for zebrafish larvae (Emran et al., 2008; MacPhail et al., 2009; Fitzgerald et al., 2019). Consequently, fewer effects were detected during light periods compared to dark periods. Diazinon-treated larvae from 2 to 24 hpf at day 4 showed an increase in
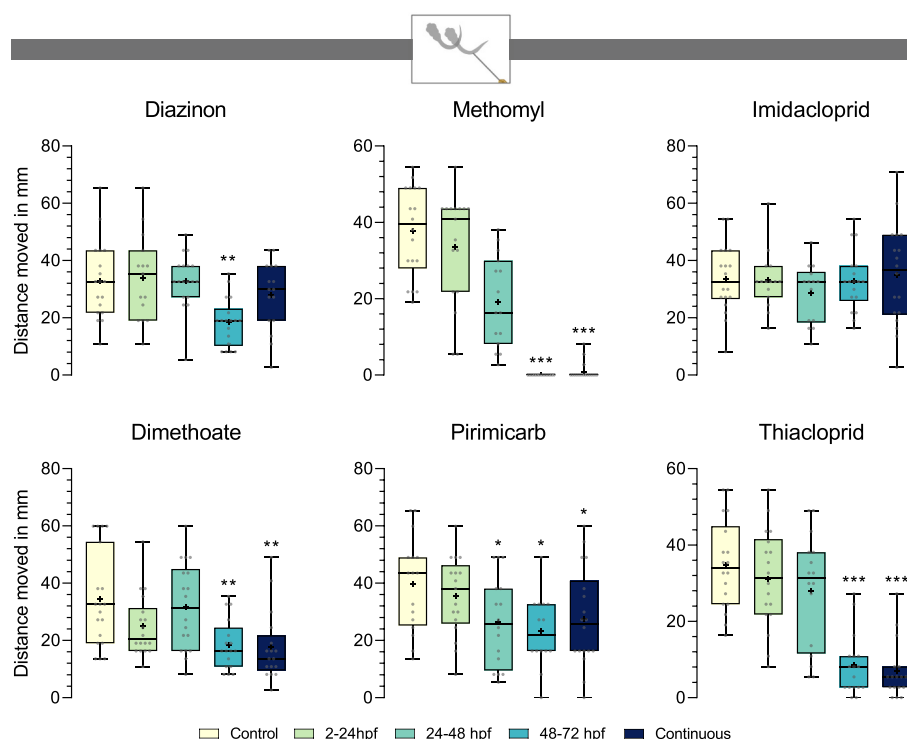
Fig. 3. Touch-evoked response expressed as distance moved in mm after insecticide exposure during different developmental periods. The boxplots show mean (cross), median (line) and minimum and maximum values as whiskers. Each dot represents one fish. Welch ANOVA with Dunnett's multiple comparisons test or Kruskal-Wallis with Dunn's multiple comparisons test was used to evaluate the statistical difference between the control and the insecticide treatments. The p values of the post-hoc test are * = $p \leq 0.033$, ** = $p \leq 0.002$, and *** = $p \leq 0.001$. The exact p values calculated by GraphPad Prism are shown in Table S7.
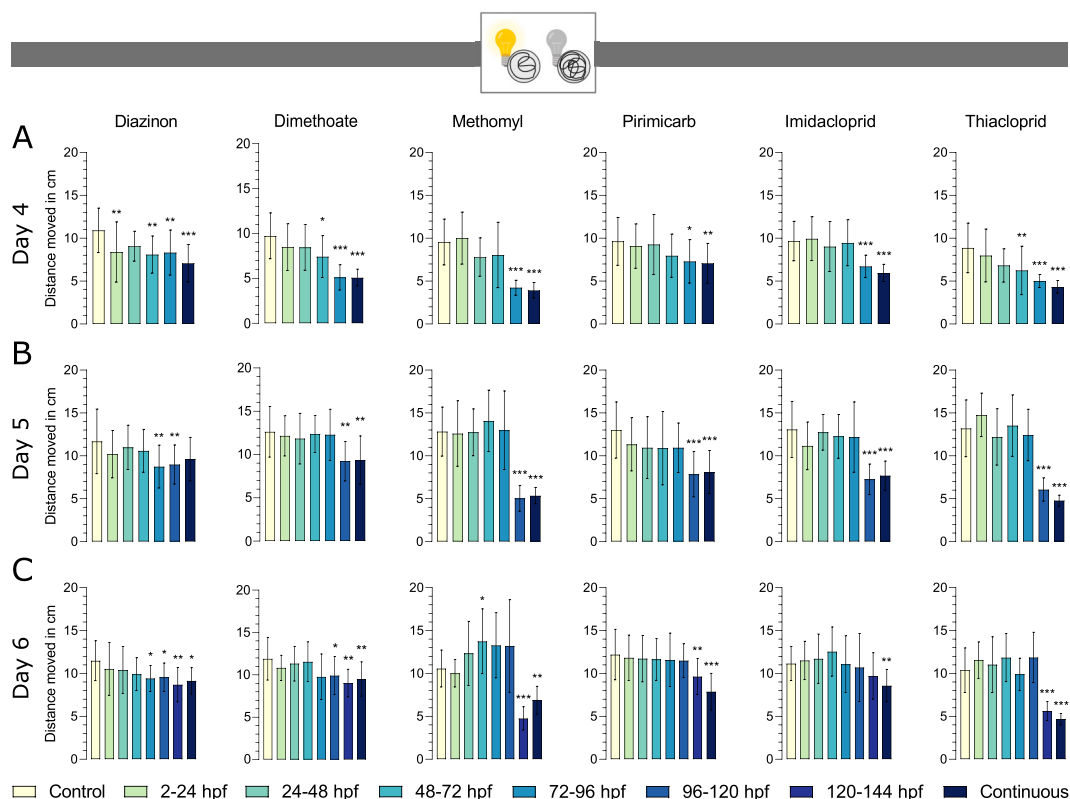
Fig. 4. Locomotion during dark phases measured on day 4 (A), day 5 (B) and day 6 (C) shown as distance moved in cm. Larvae were exposed continuously or during different developmental periods. Welch ANOVA (alpha = 0.05) with Dunnett's multiple comparisons test or Kruskal-Wallis with Dunn's multiple comparisons test was used to evaluate the statistical difference between the control and the insecticide treatments. The p values are * = p ≤ 0.033, ** = p ≤ 0.002, and *** = p ≤ 0.001. Error bars show the standard deviation. The exact p values calculated by GraphPad Prism are shown in Tables S8, S9 and S10.

distance moved of 14% compared to the control group in the light period. At day 6, larvae exposed to diazinon from 48 to 72 hpf showed a reduction in locomotion of 16% during light periods. Dimethoate-exposed larvae showed a reduction in locomotion at day 5 when exposed from 96 to 120 hpf as well as when exposed from 72 to 96 or 120 to 144 hpf at day 6 during light periods compared to the control group. For larvae exposed to thiacloprid during 48–72 hpf, we could observe a reduction in distance moved at day 4 and day 5 as well as a reduction for continuously exposed larvae at day 6. Larvae continuously exposed to imidacloprid also showed a reduction in locomotion at day 5 and 6. As well, imidacloprid exposure from 96 to 120 hpf led to reduced locomotion at day 5 compared to the respective control group.

Generally, we observed a stronger reduction for exposed larvae in the dark periods as compared to light periods, with the most drastic effects for continuous exposures as well as exposures during the 24 h preceding the light-dark transition assay.

4. Discussion

In this study, acute exposure to different cholinergic insecticides during separate 24-h windows of zebrafish neurodevelopment induced changes in larval spontaneous tail coiling, touch-evoked response, and locomotion (Fig. 5). The observed effects were more pronounced when the exposure windows were temporally closer to the performing of the
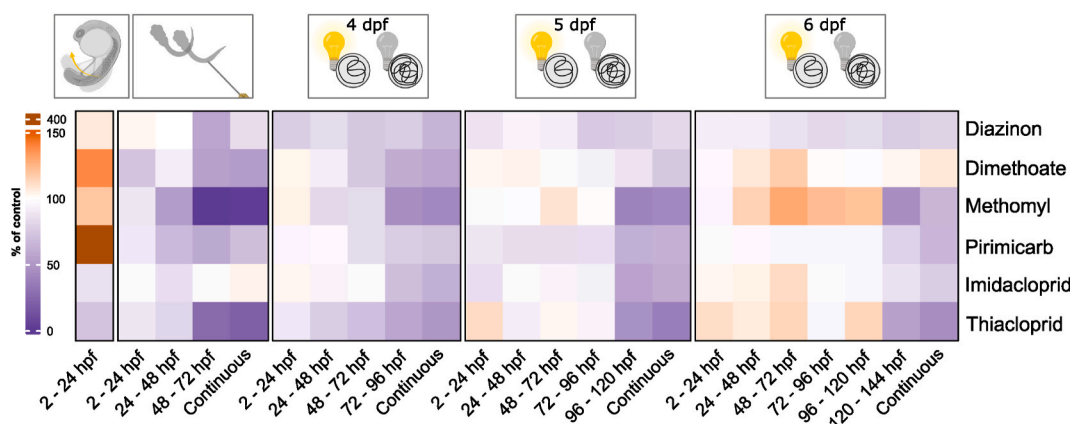
Fig. 5. Heat map summarizing the results for the different endpoints tested. Values were normalized to the respective control and are therefore shown as a percentage of the control. White boxes indicate effects that are similar to the control (100% of control), while violet and orange boxes show effects below and above control level, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

respective behavioral assay. For the cholinergic insecticides at the tested concentrations, none of the exposure windows were identified as particularly critical for locomotor performance at later larval stages, as early occurring effects did not persist in later stages. Further, by linking the outcomes of the different behavioral assays, we could see that there is no direct concordance between locomotor activity observed during early development (spontaneous tail coiling) and at later stages (TER and LDT), i.e. in some cases the same chemical at the same concentration induced hyper-or hypoactivity depending on the stage of development.

4.1. Spontaneous tail coiling was differently affected

As the earliest endpoints, the number and duration of spontaneous tail coils were assessed at 24 hpf. These spontaneous movements occur during neuro- and synaptogenesis (from 17 to 26 hpf) and are induced by spinal interneurons forming a spinal central pattern generator (CPG), which produces rhythmic depolarizations followed by unilateral muscle contractions (Brustein et al., 2003; Tong and McDearmid, 2012, Warp et al., 2012). Therefore, these endpoints can be used to check the integrity of the circuit underlying these movements, which includes a set of interneurons, motor neurons, as well as muscles. In this study, we found that the carbamate insecticides, pirimicarb and methomyl, and the organophosphate, dimethoate, all of which cause inhibition of the acetylcholinesterase, induced an increase in the number of tail coils. These results are in line with a recent study by Ogungbemi and colleagues, who have shown that several AChE inhibitors, such as chlorpyrifos, diazinon, paraoxon-methyl, and aldicarb, caused hyperactivity in the tail coiling assay (Ogungbemi et al., 2020). However, for diazinon, despite it being an AChE inhibitor as well, we did not detect a significant effect on tail coiling at the concentration tested. This is in contrast to the findings of Ogungbemi et al. (2020), who tested lower concentrations (<5 μM), but in accordance with two other studies which have shown that very similar concentrations of diazinon (~3 mg/L) did not affect tail coiling in zebrafish larvae (Velki et al., 2017a; Watson et al., 2014). These differences in the phenotypes observed can mainly be attributed to the range of exposure concentrations selected in the different studies, based on which the chemical either induced hypoactivity or hyperactivity. Such biphasic behavioral responses have previously been described (Akhtar et al., 2013; Ogungbemi et al., 2020). In addition, the uptake, metabolism, and elimination of the chemical has to be taken into account: The chorion has been shown to function as a barrier for chemical uptake, thereby influencing whether the chemical can reach the embryo or not (Henn and Braunbeck, 2011). Based on the small sizes of the chemicals tested here (all between 200 and 300 Da), it is unlikely that the chorion blocks their uptake (Pelka et al., 2017). However, for diazinon with a $\log K_{ow} > 3.4$, we cannot exclude decreased chorion passage due to interaction of the chemical with the chorion. Since in our study as well as the other studies mentioned the chorion has not been removed, this aspect does not explain the differences in the tail coiling activity. Regarding biotransformation in zebrafish, it has been shown that different developmental stages vary in their metabolic capacities when it comes to either quantity of formed metabolites or the biotransformation pathways used (Loerracher and Braunbeck, 2021). This dynamic of different metabolites present at different times of the development may also be responsible for differences in observed effects. Furthermore, we have tested the effect of two neonicotinoids, imidacloprid and thiacloprid, on the spontaneous tail coiling activity. Despite the common mode of action (nAChR agonism) and the structural similarity, we observed that imidacloprid did not affect tail coiling, while thiacloprid exposure led to a reduction in the number of tail coils measured. Our result for imidacloprid is in accordance with previous findings, which have shown that imidacloprid caused neither hyperactivity nor hypoactivity (Ogungbemi et al., 2020). For thiacloprid and acetamiprid, another neonicotinoid, it has been shown that comparable concentrations reduced spontaneous tail coiling as well (Ma et al., 2019; Von Hellfeld et al., 2022). A possible explanation for the different

phenotypes caused by imidacloprid and thiacloprid could lie in the binding affinity to the nAChR, which is lower for imidacloprid (Tomizawa and Casida, 2005). As previously mentioned, the spontaneous tail coiling assay can be applied to monitor the development and integrity of the circuit that is responsible for the generation of spontaneous movements. At the time we assessed this behavior in this study (24 hpf), the coiling circuit comprised a set of glutamatergic and glycinergic interneurons as well as the cholinergic primary motor neurons (Saint-Amant et al., 2010). As the tested insecticides primarily interfere with the cholinergic system, one would expect that alterations in tail coiling are mediated by cholinergic motor neurons. For thiacloprid, which acts as an agonist of the nAChR, it is possible that the effect on spontaneous tail coiling is directly caused by activation of nAChRs. It has been shown in previous studies that nAChRs are present early in development and that e.g. the administration of nicotine modulates spontaneous motor output (Thomas et al., 2009). However, with regard to AChE inhibitors it has been demonstrated by Yozzo et al. (2013), who investigated the effect of paraoxon on spontaneous coiling, that the observed increase in coiling frequency may not be associated with AChE inhibition, which is based on their findings showing that at this developmental stage AChE activity is very low. They further show that ACh levels are very low at this stage of embryonic development, suggesting that AChE inhibition may not have the same severe or, in fact, may even have the opposite effects to those in older larvae. Further, it is more likely that AChE inhibitors, such as carbamates and organophosphates, act via non-cholinergic mechanisms. One such mechanism has been suggested by Drapeau and colleagues (2000 and 2001), who showed that after inhibition of chemical neurotransmission with botulinum toxin, spontaneous movements were unaffected, while blocking of the gap junction (electrical synapse) induced inhibition of spontaneous movements (Saint-Amant and Drapeau, 2001, Brustein et al., 2003). Therefore, alterations in spontaneous movements elicited by pirimicarb, methomyl and dimethoate may be caused by structural or functional impairment of gap junctions. This explanation is further supported by a study on the mechanisms of toxicity of organophosphate pesticides, which has shown that non-cholinergic neurochemical processes may be affected as well (Pope, 1999, Jacobson et al., 2010), which also may be the case for carbamate insecticides.

4.2. Insecticides mostly reduced touch-evoked locomotion

Examining the development of the zebrafish locomotor network and the progression of motor behaviors, we further assessed how insecticide exposure influenced the touch-evoked response (TER) of zebrafish larvae at 72 hpf. At this age, larvae are able to swim in response to a tactile stimulus, which requires functional Rohon-Beard sensory neurons, hindbrain reticulospinal neurons, spinal cord interneurons and secondary motor neurons (Brustein et al., 2003). Consequently, an impairment in the functionality of one of these structures can ultimately lead to a change in the TER. For the insecticides tested in our study, we observed that most of them led to a decrease in touch-evoked response, which is in accordance with the results of previous studies. For example, it has been shown for the organophosphates phosmet, methamidophos and chlorpyrifos-oxon that exposed zebrafish larvae showed a decrease in TER up to the point of complete loss of the response (Yang et al., 2011; He et al., 2017; Vasamsetti et al., 2020). Further, it has also been reported that the neonicotinoid acetamiprid elicited a reduction in the TER in dechorionated fish at 48 hpf (Ma et al., 2019), which is in line with our results for thiacloprid. Similarly, we would have expected imidacloprid exposure to likewise lead to an altered TER. The absence of an effect may also be due to the lower binding affinity of imidacloprid. Comparing the TER with the activity in the spontaneous tail coiling assay, it is striking that the AChE inhibiting insecticides pirimicarb, methomyl and dimethoate induced an increase in spontaneous tail coiling but then a decrease in TER. This difference in the phenotypes observed may be related to the differences in cellular mechanisms

underlying the two motor behaviors. While spontaneous tail coiling is based on signal transmission via electrical coupling (gap junctions), touch-evoked swimming is based on chemical synaptic inputs, which means that signal transmission there largely relies on neurotransmitters. This implies that acetylcholine as well as AChE play a more important role in the generation of TER, which in turn also means that interference with the cholinergic system has more severe effects. Due to the general importance of cholinergic signaling for neuronal and muscular development, the effects connected to AChE inhibition are manifold and can include defects in Rohon-Beard neuron development or functioning, alterations in axonal length and branching, neuromuscular junction discontinuities, and affected muscle integrity (Behra et al., 2002; Jacobson et al., 2010; Pullaguri et al., 2021). Based on the results of our study, defective Rohon-Beard neurons can rather be excluded because exposed larvae were able to perceive the stimulus, but then failed to swim away. This rather suggests effects further downstream in the circuit, e.g. in interneurons, motor neurons or muscles. For methomyl and thiacloprid, we have shown in a previous study that decreased locomotion may be related to impaired muscle development as well as aberrant axon development in methomyl-exposed fish (Könemann et al., 2022). In that previous study, we have also shown that zebrafish larvae are able to recover from insecticide-induced neuromuscular and neurobehavioral changes. This is consistent with the results of the present study, in which significant behavioral effects were only measured for exposure windows temporally close to endpoint assessment. The longer the interval between exposure and endpoint assessment, the more similar control and insecticide-treated fish behaved. Such rapid recovery has also been demonstrated for other neuroactive chemicals, such as paraoxon and abermectin, and might be attributed to the plasticity of the zebrafish nervous system at larval stages (Ohnmacht et al., 2016). However, it might be possible that changes in molecular events, e.g. gene expression or neurotransmitter level changes, occurred, which we have not captured here (Schüttler et al., 2017; Tufi et al., 2016; Velki et al., 2017b). Such changes might require longer recovery or prevent recovery entirely, which might have long-term consequences for the organism. Several studies have even shown effects of exposure in following generations which in some cases even exceeded the toxicity in F0 (Schmitt et al., 2020, Blanc et al., 2021; Pompermaier et al., 2022).

4.3. The light-dark response only is reduced in presence of insecticides

Upon inflation of the swim bladder, at around days 3–4 (96 hpf), zebrafish larvae transition from burst swimming to beat-and-glide swimming, a motor behavior that is characterized by wave-like caudally spreading contractions (beats) followed by glides (Buss and Drapeau, 2001). Beat-and-glide swimming requires interaction of the hindbrain with the spinal cord and is further based on glutamatergic and glycinergic input to the motor neurons. This swimming pattern then becomes sustained at day 4–5 with the appearance of aminergic neuromodulators, such as serotonin (Drapeau et al., 2002). In the light-dark transition assay, which we used to assess the locomotor behavior of larvae, sub-lethal exposure mostly induced a reduction in locomotion. Compromised locomotion has been previously reported for different AChE-inhibiting and nAChR-activating chemicals and is caused by over-excitation of the postsynapse, which can eventually lead to paralysis and death of the organism (Russom et al., 2014; Crosby et al., 2015; Ma et al., 2019). Similar to the results of the TER assay, we were able to observe a pattern emerging in which the continuous exposure as well as the latest exposure window prior to behavior assessment induced significant effects. For larvae affected by exposure at earlier stages, locomotion returned to control levels by day 6 and even tended to exceed them in the case of methomyl (shown as orange colored boxes in the methomyl line at day 6 in Fig. 5). As a second pattern, it can be observed that in the course of locomotion assessment from day 4 to day 5 to day 6, earlier exposure first elicited hypoactivity (day 4), which then changed to hyperactivity on day 6 (Figs. 4 and 5). Both of these patterns

demonstrate the ability of larvae to recover from previous effects caused by insecticides at the concentrations tested. Further, with regard to the critical exposure windows, our data indicates that for the insecticides and concentrations tested none of the early occurring effects had an impact on locomotor performance at 6 dpf. Hence, in this case, the selected exposure windows were not found to be critical for development of the locomotor system. This outcome is in accordance with a study by Yozzo et al. (2013), who have investigated the effect of paraoxon (organophosphate) on different developmental stages and did not identify any particularly sensitive periods. In another study, however, a period between 0 and 4 hpf has been identified as being most sensitive after zebrafish embryos were exposed to the AChE inhibitor azinphos-methyl (Massei et al., 2015). Further, it has been shown that zebrafish exposed to ethanol during *prim-6* and *prim-16* were most susceptible, with pharyngeal arch hypoplasia and behavioral impairment being more common than at later stages (Ali et al., 2011). Moreover, a sensitive window of 8–48 hpf has been identified when zebrafish embryos were exposed to the flame retardant tetrabromobisphenol A (Chen et al., 2016). Based on all of these studies showing critical windows of exposure for most of the chemicals, it is rather unlikely that exposure to the insecticides tested is equally critical throughout all developmental stages. It is, however, much more likely that we have failed to properly capture relevant but much more subtle phenotypic modifications. As described by Burggren and Mueller (2015), only a small fraction of possible phenotypical modifications have been revealed to date. As possible reasons for this they have mentioned, for example, the choice of high stressor concentrations, an overly rigid definition of critical window borders, and an inordinately late definition of the onset of the critical window. As a consequence, the critical window observed is much smaller than the actual critical window. This observation is also supported by other studies that have described severe impairment of more complex behaviors such as learning, memory or social behavior in adult fish resulting from developmental exposure (Sledge et al., 2011). For future research, it may therefore be of interest to further delve into critical window assessment of the insecticides tested by extending the concentration range, the borders of critical windows, and by including adult behavior as well. Moreover, an assessment of changes at the molecular level might give further hints as to the biological processes affected by different developmental exposure windows.

4.4. Relevance for the environment and risk assessment

Environmental insecticide concentrations measured in high-income countries usually are in the ng/L to μg/L range. However, due to higher application rates combined with inappropriate application, in low-income countries in the global south, insecticides can occur at concentrations in the low mg/L range (Weiss et al., 2016). The concentrations tested in this study represent a worst-case scenario and therefore exceed environmental concentrations occurring in Europe and North America. Therefore, the observed effects are unlikely to occur at environmentally relevant concentrations. In an environmental context, however, several additional factors, not covered in this study, have to be considered. For example, in the environment, chemicals are present as complex mixtures and therefore mixture toxicity, especially for those chemicals with similar modes of action, plays an important role (Jakobs et al., 2020). Furthermore, due to differences in species sensitivity, effects may occur at much lower concentrations in other fish species (Vignet et al., 2019). This may be of particular concern when tropical species, such as the zebrafish, are used as a proxy for other fish species, including cold-water species.

With regard to risk assessment, laboratory-derived behavioral data has been shown to be environmentally relevant as it can be linked to effects on individual as well as population and ecosystem level (Ford et al., 2021). However, our studies rather had a fundamental focus on improving the understanding of the mechanisms underlying locomotion defects and therefore focused on higher concentrations representing

worst-case exposure scenarios. Consequently, the results obtained with the present study can be primarily used as background information in regulation to support the identification of hazard concerns. Particularly interesting was the observation that the results of the spontaneous tail coiling test did not correspond to the effects observed in the touch-evoked response test and the light-dark response test. This actually shows that the spontaneous tail coiling test, which has been proposed as an early warning tool to identify neurotoxic chemicals, should not be used as the only indicator.

5. Conclusion

In conclusion, this study shows that the concentrations of insecticides tested induced effects on spontaneous tail coiling, touch-evoked response and locomotion. More precisely, we show that different phenotypes emerged during different developmental stages as when, for example, AChE inhibitors which initially induced hyperactivity, later transitioned to hypoactivity. This indicates that the outcome of a later endpoint cannot be easily inferred from an earlier phenotype. Under the conditions (single substances, single exposures) tested, we did not identify any critical windows. A more environmentally realistic exposure scenario including mixtures and repeated exposures might be interesting to look into in the future. As our results rather provide fundamental insights into the effects of insecticides on fish, they cannot be directly used in risk assessment to derive effect or threshold values. However, the results can be used as valuable background information and base for following studies as they indicate that all developmental stages are affected as long as insecticides are present. Since, particularly in agriculturally influenced water bodies, insecticides and other chemicals are almost always present, the locomotion of fish can be affected, which potentially reduces the number of offspring (easier prey) and negatively affects survival of the whole population.

Credit author statement

Melissa von Wyl: Investigation, Formal analysis, Writing – original draft; **Sarah Könemann**: Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Supervision; **Colette vom Berg**: Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data is available under https://doi.org/10.25678/000618.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.137874.

References

Akhtar, M.T., Ali, S., Rashidi, H., van der Kooy, F., Verpoorte, R., Richardson, M.K., 2013. Developmental effects of cannabinoids on zebrafish larvae. Zebrafish 10 (3), 283–293.

Ali, S., Champagne, D.L., Alia, A., Richardson, M.K., 2011. Large-scale analysis of acute ethanol exposure in zebrafish development: a critical time window and resilience. PLoS One 6 (5), e20037.

Ankley, G.T., Johnson, R.D., 2004. Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. ILAR J. 45 (4), 469–483.

Arevalo, E., Lassalle, G., Tétard, S., Maire, A., Sauquet, E., Lambert, P., Paumier, A., Villeneuve, B., Drouineau, H., 2020. An innovative bivariate approach to detect joint temporal trends in environmental conditions: application to large French rivers and diadromous fish. Sci. Total Environ. 748, 141260.

Barone Jr., S., Das, K., Lassiter, T., White, L., 2000. Vulnerable processes of nervous system development: a review of markers and methods. Neurotoxicology 21 (1–2), 15–36.

Behra, M., Cousin, X., Bertrand, C., Vonesch, J.-L., Biellmann, D., Chatonnet, A., Strähle, U., 2002. Acetylcholinesterase is required for neuronal and muscular development in the zebrafish embryo. Nat. Neurosci. 5 (2), 111–118.

Bernhardt, E.S., Rosi, E.J., Gessner, M.O., 2017. Synthetic chemicals as agents of global change. Front. Ecol. Environ. 15 (2), 84–90.

Blanc, M., Antczak, P., Cousin, X., Grunau, C., Scherbak, N., Rüegg, J., Keiter, S.H., 2021. The insecticide permethrin induces transgenerational behavioral changes linked to transcriptomic and epigenetic alterations in zebrafish (Danio rerio). Sci. Total Environ. 779, 146404.

Brack, W., Barcelo Culleres, D., Boxall, A.B.A., Budzinski, H., Castiglioni, S., Covaci, A., Dulio, V., Escher, B.I., Fantke, P., Kandie, F., Fatta-Kassinos, D., Hernández, F.J., Hilscherová, K., Hollender, J., Hollert, H., Jahnke, A., Kasprzyk-Hordern, B., Khan, S.J., Kortenkamp, A., Kümmerer, K., Lalonde, B., Lamoree, M.H., Levi, Y., Lara Martín, P.A., Montagner, C.C., Mougin, C., Msagati, T., Oehlmann, J., Posthuma, L., Reid, M., Reinhard, M., Richardson, S.D., Rostkowski, P., Schymanski, E., Schneider, F., Slobodnik, J., Shibata, Y., Snyder, S.A., Fabriz Sodré, F., Teodorovic, I., Thomas, K.V., Umbuzeiro, G.A., Viet, P.H., Yew-Hoong, K.G., Zhang, X., Zuccato, E., 2022. One planet: one health. A call to support the initiative on a global science–policy body on chemicals and waste. Environ. Sci. Eur. 34 (1), 21.

Brustein, E., Saint-Amant, L., Buss, R.R., Chong, M., McDearmid, J.R., Drapeau, P., 2003. Steps during the development of the zebrafish locomotor network. J. Physiol. Paris 97 (1), 77–86.

Burggren, W.W., Mueller, C.A., 2015. Developmental critical windows and sensitive periods as three-dimensional constructs in time and space. Physiol. Biochem. Zool. 88 (2), 91–102.

Buss, R.R., Drapeau, P., 2001. Synaptic drive to motoneurons during fictive swimming in the developing zebrafish. J. Neurophysiol. 86 (1), 197–210.

Cao, F., Souders, C.L., Li, P., Pang, S., Qiu, L., Martyniuk, C.J., 2018. Biological impacts of organophosphates chlorpyrifos and diazinon on development, mitochondrial bioenergetics, and locomotor activity in zebrafish (Danio rerio). Neurotoxicol. Teratol. 70, 18–27.

Chen, J., Tanguay, R.L., Xiao, Y., Haggard, D.E., Ge, X., Jia, Y., Zheng, Y., Dong, Q., Huang, C., Lin, K., 2016. TBBPA exposure during a sensitive developmental window produces neurobehavioral changes in larval zebrafish. Environ. Pollut. 216, 53–63.

Crosby, E.B., Bailey, J.M., Oliveri, A.N., Levin, E.D., 2015. Neurobehavioral impairments caused by developmental imidacloprid exposure in zebrafish. Neurotoxicol. Teratol. 49, 81–90.

Drapeau, P., Saint-Amant, L., Buss, R.R., Chong, M., McDearmid, J.R., Brustein, E., 2002. Development of the locomotor network in zebrafish. Prog. Neurobiol. 68 (2), 85–111.

Emran, F., Rihel, J., Dowling, J.E., 2008. A behavioral assay to measure responsiveness of zebrafish to changes in light intensities. JoVE 20.

Fitzgerald, J.A., Kirla, K.T., Zinner, C.P., vom Berg, C.M., 2019. Emergence of consistent intra-individual locomotor patterns during zebrafish development. Sci. Rep. 9 (1), 13647.

Fitzgerald, J.A., Könemann, S., Krümpelmann, L., Županič, A., vom Berg, C., 2021. Approaches to test the neurotoxicity of environmental contaminants in the zebrafish model: from behavior to molecular mechanisms. Environ. Toxicol. Chem. 40 (4), 989–1006.

Ford, A.T., Ågerstrand, M., Brooks, B.W., Allen, J., Bertram, M.G., Brodin, T., Dang, Z., Duquesne, S., Sahm, R., Hoffmann, F., 2021. The role of behavioral ecotoxicology in environmental protection. Environ. Sci. Technol. 55 (9), 5620–5628.

Groh, K., vom Berg, C., Schirmer, K., Tlili, A., 2022. Anthropogenic chemicals as underestimated drivers of biodiversity loss: scientific and societal implications. Environ. Sci. Technol. 56 (2), 707–710.

Guzman, L., Besa, G., Linares, D., González, L., Pont, C., Bartolini, M., Haigis, A.-C., Legradi, J., Muñoz-Torrero, D., Gómez-Catalán, J., Barenys, M., 2020. Evaluation of the effects of acetylcholinesterase inhibitors in the zebrafish touch-evoked response: quantitative vs. qualitative assessment. Environ. Sci. Eur. 32 (1), 145.

He, X., Gao, J., Dong, T., Chen, M., Zhou, K., Chang, C., Luo, Q., Wang, C., Wang, S., Chen, D., Zhou, Z., Tian, Y., Xia, Y., Wang, X., 2017. Developmental neurotoxicity of methamidophos in the embryo-larval stages of zebrafish. Int. J. Environ. Res. Publ. Health 14 (1), 23.

Henn, K., Braunbeck, T., 2011. Dechorionation as a tool to improve the fish embryo toxicity test (FET) with the zebrafish (Danio rerio). Comp. Biochem. Physiol. C Toxicol. Pharmacol. 153 (1), 91–98.

Jacobson, S.M., Birkholz, D.A., McNamara, M.L., Bharate, S.B., George, K.M., 2010. Subacute developmental exposure of zebrafish to the organophosphate pesticide

metabolite, chlorpyrifos-oxon, results in defects in Rohon-Beard sensory neuron development. Aquat. Toxicol. 100 (1), 101–111.

Jakobs, G., Krüger, J., Schüttler, A., Altenburger, R., Busch, W., 2020. Mixture toxicity analysis in zebrafish embryo: a time and concentration resolved study on mixture effect predictivity. Environ. Sci. Eur. 32 (1), 143.

Jarema, K.A., Hunter, D.L., Shaffer, R.M., Behl, M., Padilla, S., 2015. Acute and developmental behavioral effects of flame retardants and related chemicals in zebrafish. Neurotoxicol. Teratol. 52, 194–209.

Jin, M., Zhang, X., Wang, L., Huang, C., Zhang, Y., Zhao, M., 2009. Developmental toxicity of bifenthrin in embryo-larval stages of zebrafish. Aquat. Toxicol. 95 (4), 347–354.

Kienle, C., Köhler, H.-R., Gerhardt, A., 2009. Behavioural and developmental toxicity of chlorpyrifos and nickel chloride to zebrafish (Danio rerio) embryos and larvae. Ecotoxicol. Environ. Saf. 72 (6), 1740–1747.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dynam. 203 (3), 253–310.

Koger, C.S., Teh, S.J., Hinton, D.E., 2000. Determining the sensitive developmental stages of intersex induction in medaka (Oryzias latipes) exposed to 17 beta-estradiol or testosterone. Mar. Environ. Res. 50 (1–5), 201–206.

Könemann, S., Meyer, S., Betz, A., Županič, A., vom Berg, C., 2021. Sub-lethal peak exposure to insecticides triggers olfaction-mediated avoidance in zebrafish larvae. Environ. Sci. Technol. 55 (17), 11835–11847.

Könemann, S., von Wyl, M., vom Berg, C., 2022. Zebrafish larvae rapidly recover from locomotor effects and neuromuscular alterations induced by cholinergic insecticides. Environ. Sci. Technol. 56 (12), 8449–8462.

Leuthold, D., Klüver, N., Altenburger, R., Busch, W., 2019. Can environmentally relevant neuroactive chemicals specifically Be detected with the locomotor response test in zebrafish embryos? Environ. Sci. Technol. 53 (1), 482–493.

Loerracher, A.-K., Braunbeck, T., 2021. Cytochrome P450-dependent biotransformation capacities in embryonic, juvenile and adult stages of zebrafish (Danio rerio)—a state-of-the-art review. Arch. Toxicol. 95 (7), 2299–2334.

Ma, X., Li, H., Xiong, J., Mehler, W.T., You, J., 2019. Developmental toxicity of a neonicotinoid insecticide, acetamiprid to zebrafish embryos. J. Agric. Food Chem. 67 (9), 2429–2436.

Maack, G., Segner, H., 2004. Life-stage-dependent sensitivity of zebrafish (Danio rerio) to estrogen exposure. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 139 (1–3), 47–55.

MacPhail, R.C., Brooks, J., Hunter, D.L., Padnos, B., Irons, T.D., Padilla, S., 2009. Locomotion in larval zebrafish: influence of time of day, lighting and ethanol. Neurotoxicology 30 (1), 52–58.

Massei, R., Vogs, C., Renner, P., Altenburger, R., Scholz, S., 2015. Differential sensitivity in embryonic stages of the zebrafish (Danio rerio): the role of toxicokinetics for stage-specific susceptibility for azinphos-methyl lethal effects. Aquat. Toxicol. 166, 36–41.

Ogungbemi, A.O., Teixido, E., Massei, R., Scholz, S., Küster, E., 2020. Optimization of the spontaneous tail coiling test for fast assessment of neurotoxic effects in the zebrafish embryo using an automated workflow in KNIME. Neurotoxicol. Teratol. 81, 106918.

Ohnmacht, J., Yang, Y., Maurer, G.W., Barreiro-Iglesias, A., Tsarouchas, T.M., Wehner, D., Sieger, D., Becker, C.G., Becker, T., 2016. Spinal motor neurons are regenerated after mechanical lesion and genetic ablation in larval zebrafish. Development 143 (9), 1464–1474.

Pelka, K.E., Henn, K., Keck, A., Sapel, B., Braunbeck, T., 2017. Size does matter–Determination of the critical molecular size for the uptake of chemicals across the chorion of zebrafish (Danio rerio) embryos. Aquat. Toxicol. 185, 1–10.

Pompermaier, A., Tamagno, W.A., Alves, C., Barcellos, L.J.G., 2022. Persistent and transgenerational effects of pesticide residues in zebrafish. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 262, 109461.

Pope, C.N., 1999. Organophosphorus pesticides: do they all have the same mechanism of toxicity? J. Toxicol. Environ. Health B Crit. Rev. 2 (2), 161–181.

Pullaguri, N., Grover, P., Abhishek, S., Rajakumara, E., Bhargava, Y., Bhargava, A., 2021. Triclosan affects motor function in zebrafish larva by inhibiting ache and syn2a genes. Chemosphere 266, 128930.

Rice, D., Barone, S., 2000. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ. Health Perspect. 108 (Suppl. 3), 511–533.

Richendrfer, H., Creton, R., Colwill, R.M., 2014. In: The Embryonic Zebrafish as a Model System to Study the Effects of Environmental Toxicants on Behavior. Zebrafish; Nova Science Publishers, New York, NY, USA, pp. 245–264.

Rima, M., Lattouf, Y., Abi Younes, M., Bullier, E., Legendre, P., Mangin, J.-M., Hong, E., 2020. Dynamic regulation of the cholinergic system in the spinal central nervous system. Sci. Rep. 10 (1), 15338.

Russom, C.L., LaLone, C.A., Villeneuve, D.L., Ankley, G.T., 2014. Development of an adverse outcome pathway for acetylcholinesterase inhibition leading to acute mortality. Environ. Toxicol. Chem. 33 (10), 2157–2169.

Saint-Amant, L., 2010. Chapter 4 - development of motor rhythms in zebrafish embryos. Prog. Brain Res. 187, 47–61. J.-P. Gossard, R. Dubuc and A. Kolta, Elsevier.

Saint-Amant, L., Drapeau, P., 1998. Time course of the development of motor behaviors in the zebrafish embryo. J. Neurobiol. 37 (4), 622–632.

Saint-Amant, L., Drapeau, P., 2001. Synchronization of an embryonic network of identified spinal interneurons solely by electrical coupling. Neuron 31 (6), 1035–1046.

Schmidt, R., Strähle, U., Scholpp, S., 2013. Neurogenesis in zebrafish – from embryo to adult. Neural Dev. 8 (1), 3.

Schmitt, C., McManus, M., Kumar, N., Awoyemi, O., Crago, J., 2019. Comparative analyses of the neurobehavioral, molecular, and enzymatic effects of organophosphates on embryo-larval zebrafish (Danio rerio). Neurotoxicol. Teratol. 73, 67–75.

Schmitt, C., Peterson, E., Willis, A., Kumar, N., McManus, M., Subbiah, S., Crago, J., 2020. Transgenerational effects of developmental exposure to chlorpyrifos-oxon in zebrafish (DANIO RERIO). Toxicol. Appl. Pharmacol. 408, 115275.

Schulz, R., 2004. Field studies on exposure, effects, and risk mitigation of aquatic nonpoint-source insecticide pollution: a review. J. Environ. Qual. 33 (2), 419–448.

Schüttler, A., Reiche, K., Altenburger, R., Busch, W., 2017. The transcriptome of the zebrafish embryo after chemical exposure: a meta-analysis. Toxicol. Sci. 157 (2), 291–304.

Selderslaghs, I.W.T., Hooyberghs, J., De Coen, W., Witters, H.E., 2010. Locomotor activity in zebrafish larvae: a new method to assess developmental neurotoxicity. Neurotoxicol. Teratol. 32 (4), 460–471.

Sledge, D., Yen, J., Morton, T., Dishaw, L., Petro, A., Donerly, S., Linney, E., Levin, E.D., 2011. Critical duration of exposure for developmental chlorpyrifos-induced neurobehavioral toxicity. Neurotoxicol. Teratol. 33 (6), 742–751.

Spycher, S., Mangold, S., Doppler, T., Junghans, M., Wittmer, I., Stamm, C., Singer, H., 2018. Pesticide risks in small streams—how to get as close as possible to the stress imposed on aquatic organisms. Environ. Sci. Technol. 52 (8), 4526–4535.

Standardization, I.O.f., 1999. Water Quality: Determination of Acute Lethal Toxicity to Marine Copepods (Copepoda, Crustacea). ISO.

Stehr, C.M., Linbo, T.L., Incardona, J.P., Scholz, N.L., 2006. The developmental neurotoxicity of fipronil: notochord degeneration and locomotor defects in zebrafish embryos and larvae. Toxicol. Sci. 92 (1), 270–278.

Thomas, L.T., Welsh, L., Galvez, F., Svoboda, K.R., 2009. Acute nicotine exposure and modulation of a spinal motor circuit in embryonic zebrafish. Toxicol. Appl. Pharmacol. 239 (1), 1–12.

Tomizawa, M., Casida, J.E., 2005. Neonicotinoid insecticide toxicology: mechanisms of selective action. Annu. Rev. Pharmacol. Toxicol. 45, 247–268.

Tong, H., McDearmid, Jonathan R., 2012. Pacemaker and plateau potentials shape output of a developing locomotor network. Curr. Biol. 22 (24), 2285–2293.

Tufi, S., Leonards, P., Lamoree, M., de Boer, J., Legler, J., Legradi, J., 2016. Changes in neurotransmitter profiles during early zebrafish (Danio rerio) development and after pesticide exposure. Environ. Sci. Technol. 50 (6), 3222–3230.

van Aerle, R., Pounds, N., Hutchinson, T.H., Maddix, S., Tyler, C.R., 2002. Window of sensitivity for the estrogenic effects of ethinylestradiol in early life-stages of fathead minnow, Pimephales promelas. Ecotoxicology 11 (6), 423–434.

Vasamsetti, B.M.K., Kim, N.S., Chon, K., Park, H.-H., 2020. Developmental toxic effects of phosmet on zebrafish (Danio rerio) embryos. Korean J Pestic Sci 24, 343–351.

Velki, M., Di Paolo, C., Nelles, J., Seiler, T.-B., Hollert, H., 2017a. Diuron and diazinon alter the behavior of zebrafish embryos and larvae in the absence of acute toxicity. Chemosphere 180, 65–76.

Velki, M., Meyer-Alert, H., Seiler, T.-B., Hollert, H., 2017b. Enzymatic activity and gene expression changes in zebrafish embryos and larvae exposed to pesticides diazinon and diuron. Aquat. Toxicol. 193, 187–200.

Vignet, C., Cappello, T., Fu, Q., Lajoie, K., De Marco, G., Clérandeau, C., Mottaz, H., Maisano, M., Hollender, J., Schirmer, K., Cachot, J., 2019. Imidacloprid induces adverse effects on fish early life stages that are more severe in Japanese medaka (Oryzias latipes) than in zebrafish (Danio rerio). Chemosphere 225, 470–478.

Von Hellfeld, R., Ovcharova, V., Bevan, S., Lazaridi, M.-A., Bauch, C., Walker, P., Bennekou, S.H., Forsby, A., Braunbeck, T., 2022. Zebrafish embryo neonicotinoid developmental neurotoxicity in the FET test and behavioral assays. ALTEX 39 (3).

Warp, E., Agarwal, G., Wyart, C., Friedmann, D., Oldfield, Claire S., Conner, A., Del Bene, F., Arrenberg, Aristides B., Baier, H., Isacoff, Ehud Y., 2012. Emergence of patterned activity in the developing zebrafish spinal cord. Curr. Biol. 22 (2), 93–102.

Watson, F.L., Schmidt, H., Turman, Z.K., Hole, N., Garcia, H., Gregg, J., Tilghman, J., Fradinger, E.A., 2014. Organophosphate pesticides induce morphological abnormalities and decrease locomotor activity and heart rate in Danio rerio and Xenopus laevis. Environ. Toxicol. Chem. 33 (6), 1337–1345.

Weiss, F.T., Leuzinger, M., Zurbrügg, C., Eggen, H.I., 2016. Chemical pollution in low- and middle-income countries. from. https://www.eawag.ch/en/department/sandec/publications/chemical-pollution/.

Yang, D., Lauridsen, H., Buels, K., Chi, L.-H., La Du, J., Bruun, D.A., Olson, J.R., Tanguay, R.L., Lein, P.J., 2011. Chlorpyrifos-oxon disrupts zebrafish axonal growth and motor behavior. Toxicol. Sci. 121 (1), 146–159.

Yen, J., Donerly, S., Levin, E.D., Linney, E.A., 2011. Differential acetylcholinesterase inhibition of chlorpyrifos, diazinon and parathion in larval zebrafish. Neurotoxicol. Teratol. 33 (6), 735–741.

Yozzo, K.L., McGee, S.P., Volz, D.C., 2013. Adverse outcome pathways during zebrafish embryogenesis: a case study with paraoxon. Aquat. Toxicol. 126, 346–354.