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Chronic toxicity testing including transcriptomics-based molecular profiling in *Cloeon dipterum*

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Abstract

The so-called EPT taxa have been shown to be highly sensitive to various environmental pollutants. However, there are only few published studies on toxicity testing with EPT representatives and there is a particular lack of protocols for chronic toxicity testing, e.g., for integration into species sensitivity distribution (SSD) approaches. To address this gap, we performed a long-term 38-day semi-static toxicity test with the European mayfly species *Cloeon dipterum* using the insecticide fipronil as model substance. The functionality of the test system was confirmed by the high emergence rate of 85% in the control condition. We found a high sensitivity with regard to larval development with an EC_{50} of 180 ng/L and a NOEC of 38.0 ng/L after 7 days exposure. After 38 days, an LC_{50} value of 185 ng/L and an EC_{50} value of 160 ng/L for emergence (both: NOEC = 38.0 ng/L) were calculated. In a short-term 7-day toxicity test, we found a similar effect on larval development. In addition to the physiological endpoints, we examined fipronil-induced gene expression changes at the transcriptome level in this test. Our results revealed a concentration-dependent increase in the number of differentially expressed genes, as well as observed effects on larval development. Notably, we identified marker gene candidates involved in nervous system development, mirroring the known mode-of-action of fipronil in *C. dipterum*. The affected genes primarily play crucial roles in neurological processes. Concluding, within this two-step approach we were able to identify fipronil effects on the sublethal physiological endpoint larval development and to complement these effects at the molecular level by gene expression changes in the transcriptome. Thus, this assay proved to be suitable to assess sublethal effects as well as the mode-of-action of substances in the non-standard organism *C. dipterum* already after a short-term exposure of 7 days. However, further testing is required to validate the procedure.

Keywords EPT taxa, *Cloeon dipterum*, Chronic toxicity, Fipronil, Gene expression changes, Transcriptomics

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Background

For the environmental risk assessment of chemicals, toxicity testing in several aquatic organisms is required [13]. While standardized protocols are available for few aquatic invertebrates, such as *Daphnia magna* or *Chironomus* sp., which are used for tier 1 testing, there is a lack of comprehensive protocols for the aquatic larvae of EPT (Ephemeroptera, Plecoptera, Trichoptera) taxa [24–26]. These larvae, which play crucial roles in aquatic ecosystems and are involved in various ecosystem services, are frequently exposed to multiple contaminants in their environment and have been shown to be highly sensitive to various environmental pollutants [21, 30, 41]. This suggests that the current toxicity testing methods may underestimate the risks for EPT taxa, highlighting the need for adopted laboratory test protocols allowing data generation specifically for representatives of the EPT taxa. However, especially for European EPT species, there is limited literature available and standardized test protocols are lacking, particularly for chronic testing [34], to be suitable for application in species sensitivity distribution (SSD) approaches at higher tiers.

Thus, the main aim of our study was to identify a suitable test protocol for chronic toxicity testing under laboratory conditions in the mayfly *Cloeon dipterum.* The test organism was chosen due to its high abundance in water bodies in Central Europe and its short life cycle of few weeks under appropriate environmental conditions [4, 8]. Moreover, this species lives in lentic habitats, so the establishment of a flow is not required, whereas it is for most other mayfly species [6, 23, 44].

To complement classical physiological endpoints in our study, and to allow for the identification of the adverse mode-of-action (MoA) in non-target organisms, the second aim of our study was to show that transcriptomics could be integrated into laboratory protocols initially developed for chronic effect assessment. Thus, we implemented the investigation of molecular effects by analysis of gene expression changes at the transcriptome level triggered by a MoA-specific model substance like fipronil. This provides the base to identify biological processes impaired by the test substance and MoA-specific marker gene candidates to be focused on in-future screening approaches with *C. dipterum*. For genome alignment of our sequencing data, we used the transcriptome de novo assembly of *C. dipterum* published by Almudi et al. in 2019 [2]. By now, to the best of our knowledge there are no studies available investigating molecular responses to environmental pollutants in *C. dipterum* at the global gene expression level.

In our study, we employed a two-step approach to investigate the effects of the pesticide fipronil on *C. dipterum*. The first step involved a long-term exposure test lasting 38 days, during which we monitored larval development, emergence and mortality as key endpoints. We identified the earliest time point at which statistically significant sublethal effects in *C. dipterum*, such as delayed larval development, were observed in the long-term exposure test, which was 7 days, and subsequently established a 7-day-screening test in order to include transcriptomics-based assessment of molecular endpoints.

The phenylpyrazole fipronil was widely used in plant protection until its registration expired in the EU several years ago. Besides this, fipronil is still used in veterinary medicine. It acts by inhibiting γ-aminobutyric acid (GABA)-gated chloride channels, thereby disrupting the influx of chloride ions in neurons. At sufficient concentrations, this disruption causes hyperexcitation of the neuronal system of the insects, resulting in severe paralysis and death. Fipronil has a higher binding affinity to invertebrate GABA receptors than to vertebrate ones [16]. Like the group of neonicotinoids, which were previously shown to have severe effects on mayflies [21, 30, 35], fipronil acts in the neuronal system of insects. The toxicity of fipronil has been extensively investigated for various aquatic invertebrates. Cladocera were found to be rather insensitive to fipronil in comparison to different insects [11, 17, 39]. The NOEC for 28-day chronic toxicity to *Chironomus riparius* was 117 ng/L [11]. The 96-h sensitivity of the different mayfly species varied greatly with *Baetis tricaudatus* (EC_{50} = 51.9 ng/L) being most sensitive and *Serratella micheneri* (EC_{50} = 589 ng/L) being least sensitive in the endpoint swimming ability [44]. Also a 30-day mesocosm experiment in flow channels revealed high sensitivities to fipronil and its derivatives regarding the emergence of several mayfly species [23]. These data indicate that mayflies belong to the most sensitive species regarding fipronil toxicity. For these reasons, fipronil was suspected to be a suitable model substance to prove the suitability of the developed and refined laboratory chronic test in *C. dipterum*, and also to generate MoA-specific gene expression patterns for potential screening approaches (Fig. 1).

Materials and methods

Test organisms and acclimatization

The mayfly species *C. dipterum* belongs to the family of Baetidae (Ephemeroptera) and is highly abundant in Central Europe [8]. In contrast to the major proportion of mayfly species, the larvae of *C. dipterum* inhabit lentic habitats like small ponds, the littoral of lakes or slow parts of running waters, where the larvae live as typical grazers. They live on macrophytes or the sediment and feed on periphyton and detritus. Due to these properties *C. dipterum* is well suited for the use as test organism in toxicity testing. Mayflies belong to the hemimetabolic

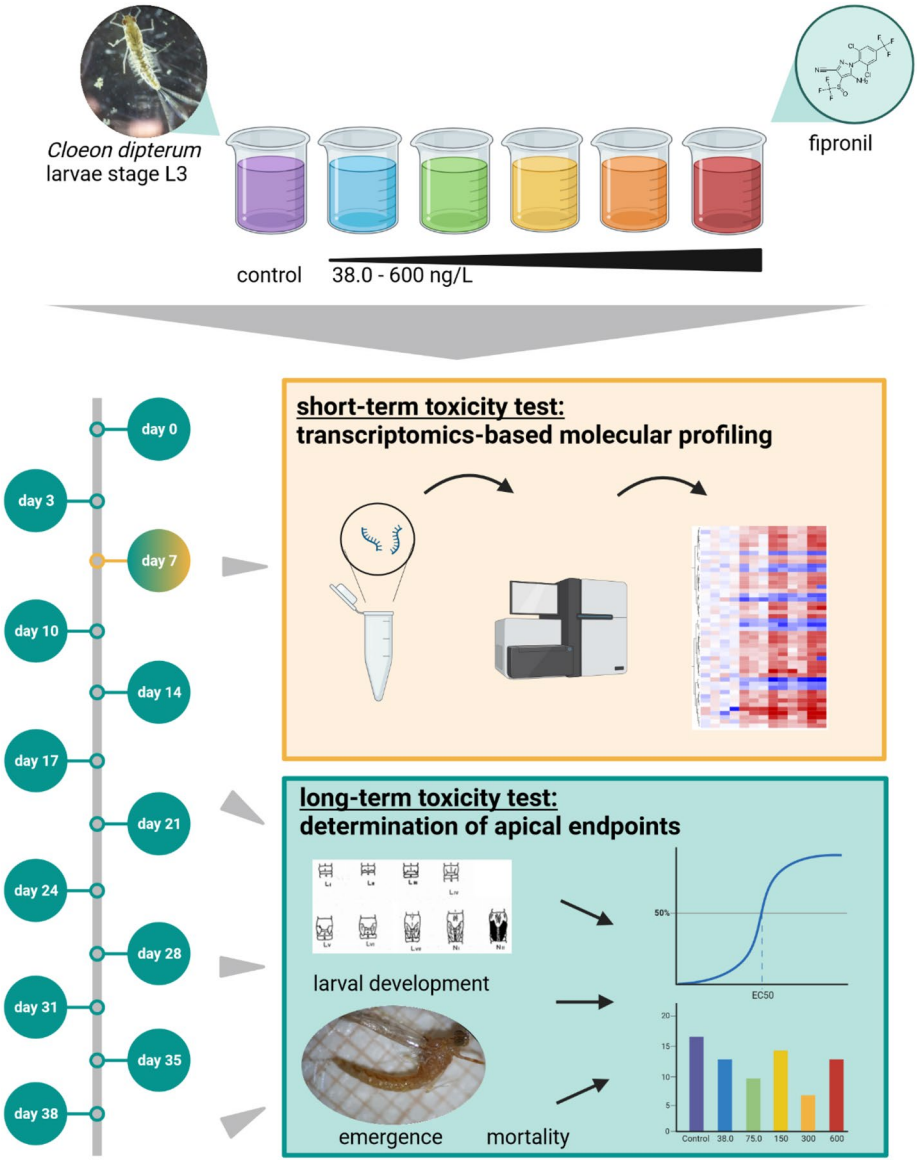

Fig. 1 Schematic representation of the long- and short-term toxicity test workflow with *C. dipterum*. Long-term toxicity test: field-collected young larvae in the larval stage L3 were exposed to a control condition and five test concentrations containing 38.0–600 ng/L fipronil in Elendt M4 medium. For each treatment, 4 replicates containing 5 larvae each were prepared. The test design was semi-static with two media renewals per week. Larvae were fed with diatoms of *N. pelliculosa* and carrots. The apical endpoints larval development, emergence and mortality were determined twice per week. The test was conducted for 38 days. Short-term exposure test: The test was conducted for a total test duration of 7 days. Three larvae per replicate and condition were used for RNA extraction, RNA sequencing-based transcriptomics and molecular profiling. (Created with BioRender.com)

insects, which go through several larval stages including various numbers of molts until (sub)imago stage. Seven larval stages (L1–L7) and two nymphe stages (N1–N2) were described for *C. dipterum*. *C. dipterum* has a bivoltine life cycle with a fast developing generation in summer and a slow developing generation in winter, thus, the duration of larval development highly depends on environmental conditions [8]. Almudi et al. [2] described a life cycle of around 40 to 60 days under laboratory conditions for *C. dipterum*. Based on previous experiments and control data, we assume that each larval stage lasts three to five days under laboratory conditions.

The larvae were caught in a small pond in the midlands of eastern North Rhine-Westphalia, Germany, close to a forest area. For this, a net was gently swiped through the macrophytes in the littoral of the pond. In beakers

containing water from the sampling site, the organisms were transferred to the laboratory, where the larvae were identified and moved into an aquarium containing pond water. They were acclimatized to the test conditions within 5 days while partially exchanging pond water with Elendt M4 medium [24, 25]. The larvae were fed with *Navicula pelliculosa* and carrots ad libitum.

At test start, the larval stage of the organisms was determined based on the wing pad development described by Cianciara in 1976 [8] and larvae in the stage L3 were introduced in each test. An overview of larval stages is given in Additional file 1: Figs. S1 and S2. Based on literature data and the sampling date, we assume that the larvae used in both tests belonged to the slow developing winter generation of *C. dipterum* [8]. However, we did not perform a regular gapless monitoring of the sampling site, therefore we are unable to determine the exact generation.

Food

C. dipterum larvae were fed with diatoms of *N. pelliculosa* grown on small tiles, and pieces of carrots. The feeding of grazing mayflies with periphyton-grown tiles was already successfully established in chronic toxicity tests with the North American species *Neocloeon triangulifer* and with the New Zealand species *Deleatidium* spp. [21, 38]. The stock culture of the *N. pelliculosa,* original strain SAG 1050-3 isolated in 1950 from a freshwater ecosystem in Connecticut, USA, was cultured in growth medium for more than ten years at Fraunhofer IME [32]. To prepare the periphyton tiles used as food source in the tests, large plastic boxes (Euroboxes, 60×40 cm) containing small ceramic tiles (4.7×4.7 cm) were filled with 5 L Elendt M4 medium, 10 mL of a nutrient solution [24, 25] (Additional file 1: Table S1) and around 100 mL of the *N. pelliculosa* stock culture as an inoculum. The diatoms were cultured for 5 to 8 days with constant illumination (25.0 to 40.0 µmol/m^2*s) at ambient temperature. For the test only evenly grown tiles, which served as food source as well as substratum for the larvae, were used.

In addition to the diatom diet, the larvae were fed with pieces of carrots, which were shown to be an appropriate food in laboratory culture of *C. dipterum* [2]. Organic carrots were cut into approximately 0.5 cm^3 pieces and cooked for around 5 min in tap water. Pieces of 0.45 to 0.55 g weight were frozen (at -18.0 °C) and defrosted immediately before use in the test.

Test substance and test solution setup

Fipronil ($C_{12}H_4Cl_2F_6N_4OS$; CAS number: 120068-37-3) was used as test substance (Additional file 1: Fig. S3). The chemical was a PESTANAL® analytical standard purchased from Sigma-Aldrich® (Batch number: BCBW 9282; purity: 98.8%).

Preliminary experiments were performed to identify a concentration range which allowed for the detection of effect concentrations for sublethal endpoints. Nominal concentrations applied in the long-term exposure test were 38.0, 75.0, 15.0, 300 and 600 ng/L. Because of high lethality at the highest test concentration, the concentrations applied in the short-term exposure test were chosen as 38.0, 75.0, 15.0 and 300 ng/L. To prepare test solutions, a stock solution of fipronil was prepared in acetone, which was further diluted with acetone to prepare the acetonic stock solutions for the lower test solutions. 1 mL of each corresponding acetonic stock solution was transferred into an empty glass bottle and distributed along the glass wall, while the bottle was turned slowly. The acetone was evaporated by blowing compressed air into the bottle and the glass bottles were filled with 2 L Elendt M4 medium each. The test solutions were treated with ultrasonication (30 min; Bandelin Sonorex RM 75 UH, BANDELIN electronic GmbH & Co. KG, 40 kHz) and stirred for 60 min at ambient temperature before application in the test.

Experimental design

Two chronic toxicity tests were performed with a similar semi-static test design, but different test durations and two media renewals per week. The wing pad development of larvae was determined using a binocular, and small larvae in the stage L3 (size: around 3–5 mm) were used for testing [8]. Cubic glass containers (14×14×14 cm) were used as test vessels, which were placed randomly in a temperature-controlled water bath. Each test vessel was filled with around 500 mL test solution resulting in a water column height of around 10 cm. At least four replicates per test concentration and control condition were used, containing five individuals per replicate. For feeding of the larvae, each test vessel contained four *N. pelliculosa*-grown tiles and a carrot piece of 0.45 to 0.55 g weight, which were replaced twice per week in parallel to test solution renewal. The tests were conducted at a temperature of 20 °C (± 1 °C) and the illumination was < 74 lux with a light and dark rhythm of 16:8 h. At test start, during each media renewal and at test end, the physico-chemical parameters pH, oxygen concentration and oxygen saturation were determined using the multiparameter device WTW Multi 1970i. The temperature was measured continuously and the light intensity was determined weekly. Water parameters are shown in Additional file 1: Table S2. Vitality and larval development were controlled twice per week during media renewal, while emergence was determined daily. If

an individual did not show any movement of gills, legs, mandibula or abdomen, it was considered dead.

In previous experiments we identified that a test duration of 38 days was sufficient to achieve an emergence of 80% in the control condition. The test vessels were covered by loose nets to prevent the escape of emerged imagines. As soon as the subimagines had molted, the sex was determined based on typical characteristics like copulation tongs and turban eyes of male mayflies and, if possible, the wing length was measured [12]. Afterwards, the emerged female imagines were sampled for further transcriptome analysis (Additional file 1: Figs. S4 to S8) by transferring a maximum of three individuals per replicate to an Eppendorf® tube containing 350 µL lysis buffer from the NucleoSpin RNA/Protein kit (Macherey & Nagel, Düren, Germany), which was subsequently frozen. Females were chosen because of their higher relevance to reproduction as compared to males. In addition, the female imagines carry out egg development, whereas male imagines mate and die shortly after. The samples were stored at approximately -18 °C until RNA extraction. In the short-term exposure experiment, the larvae of four replicates of the control condition and three replicates of each exposure condition were sampled for transcriptome analysis. Due to the lack of visible sexual characteristics, the sex of the larvae could not be determined. For each exposure condition and the control the three larvae with the lowest larval stage per replicate were transferred to an Eppendorf® tube at test end. The larvae were euthanized immediately in liquid nitrogen, and subsequently, 350 µL lysis buffer was added to the tube. RNA extraction was performed on the same day.

Statistical analysis

In both tests, the endpoints larval development and mortality were evaluated, in the long-term exposure test also the emergence was analyzed. The percentage of larvae per replicate and treatment that reached a respective larval stage was converted by arcsine transformation to create metric data for statistical analysis. The assessment of effects was based on the nominal concentrations of fipronil.

For the statistical evaluation of sublethal and lethal effects, the computer program ToxRat Professional (Version 3.3.0, 2015, ToxRat Solutions GmbH & Co. KG, Alsdorf, Germany), a statistical software package for ecotoxicity response analysis, was used. If possible, for each endpoint the no-observed-effect concentration (NOEC), the lowest-observed-effect concentration (LOEC), and EC_{10} and EC_{50} were calculated. To determine significant differences of treatments compared to the controls in the quantal data of mortality and emergence (determination of NOEC and LOEC values) the Step-down Cochran–Armitage test was used. To determine significant differences of treatments compared to the controls in the metric data of larval development (determination of NOEC and LOEC values) the Williams Multiple Sequential *t*-test was used. To define EC_x values and 95% confidence intervals, Probit Analysis assuming log-normal distribution of the values was conducted.

Total RNA isolation, transcriptome sequencing, differential gene expression analysis and overrepresentation analysis

Total RNA was isolated from imagines (long-term exposure test) and larvae (short-term exposure test) using the NucleoSpin RNA/Protein kit (Macherey & Nagel, Düren, Germany). The organisms were homogenized in lysis buffer at 5 m/s for 40 s at room temperature with Lysing Matrix D ceramic beads using a FastPrep-24 homogenizer (MP Biomedicals, Irvine, USA) before proceeding with the NucleoSpin RNA/Protein kit according to the manufacturer's instructions. The yields of total RNA isolated from each sample were quantified using a Nanodrop system (Thermo Fisher Scientific, Waltham, USA), and the RNA quality was assessed using the RNA 6000 Nano Kit on an Agilent 2100 Bioanalyzer System (Agilent, Santa Clara, USA). Poly(A) + RNA was isolated from total RNA and subjected to library preparation using the TruSeq RNA library Prep Kit v2 following the manufacturer's instructions. Libraries were sequenced in 2 × 150-bp paired-end mode on a NovaSeq 6000 system, yielding approximately 30 million raw reads per sample, which were aligned to the *C. dipterum* reference genome (GCA 902829235.1) with STAR aligner v.2.5.2a [10] and feature-mapped reads were counted using STAR's '—quantMode GeneCounts'. Differential gene expression analysis (DGEA) was performed in R v4.2.2 using DESeq2 v1.38.3 [20]. A gene was considered as differentially expressed when its mean expression differed significantly from the control group (BH adjusted p (p.adj) ≤ 0.05) and the absolute apeglm shrunk lfc was greater LFcut (abs[apeglm(lfc)] ≥ LFcut). The core DEG set was defined as the common set of identified differentially expressed genes (DEGs) of low and high exposure conditions for organisms exposed to fipronil and was evaluated using correlation analysis.

To enable functional interpretation of *C. dipterum* RNA-Seq datasets in R via clusterProfiler [7], a custom AnnotationDbi package was created following the workflow previously described by Loll et al. [19] for *Lemna minor*. Similar to this approach, the org.Cdipterum.eg.db functional gene annotation package was created based on the homology of protein sequences translated from the *C. dipterum* reference genome to a reference database.

To elucidate the biological mechanisms affected by the test substance, clusterProfiler was used to perform

enrichment analysis for GO terms for biological processes (BP) and molecular functions (MF). To identify BP and MF directly associated with the DEG sets of each sample group, an overrepresentation analysis (ORA) was performed. Detailed information about the transcriptomics workflow can be found in Additional files 1, 2 and methods.

Chemical analysis

At test start, each media renewal and test end, samples of the aqueous fresh and aged test solutions were taken and fipronil concentrations were determined by ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS). Samples were prepared by adding 5 mL of the test samples to 1 mL acetonitrile. The samples were stored at ≤ -18 °C before analysis. The limit of detection (LOD) and limit of quantification (LOQ) were 10 ng/L and 30 ng/L, respectively, and the calibration range was 10–1000 ng/L (referring to the pure aqueous portion). Detailed description of the analytical procedure, as well as chemicals and materials used, can be found in Additional file 1: Figs. S14 to S17 and methods.

Results and discussion

Long-term exposure test: fipronil effects on physiological endpoints in *C. dipterum*

The 38-day test was performed with nominal fipronil concentrations of 38.0, 75.0, 150, 300 and 600 ng/L in order to assess effects on the endpoints mortality, emergence and larval development. Chemical analysis of fipronil concentrations revealed recoveries between 92 and 126%, thus fipronil effects were evaluated based on nominal concentrations. Detailed information is given in Additional file 1: Tables S3 and S4. In the long-term exposure test, significant concentration-dependent effects were observed and determined ECx and NOEC/LOEC values are presented in Table 1. After 38 days, the mortality in the control group was 10% and ranged from 25% in concentration 38.0 ng/L to 75% in concentration 600 ng/L in the fipronil exposure groups (Additional file 1: Fig. S18). Emergence was observed first at day 14 in the control and concentrations 38.0 and 150 ng/L (Fig. 2A). At test end, the emergence in the control was 85%, while it ranged from 70% (concentration 38.0 ng/L) to 20% (concentration 600 ng/L) in the exposure conditions.

A statistical evaluation of concentration-related effects with regard to the sublethal endpoint larval development was performed for the three observation times day 3, day 7 and day 10 until first emergence was observed. After a test duration of 7 days, a statistically significant effect on the endpoint larval development was observed for the first time. 85.0% (± 19.1%) of the larvae in the control condition reached the respective larval stage L6, which was also the case in the lowest exposure concentration 38.0 ng/L, while only 12.5% (± 25%) reached this stage in the highest concentration 600 ng/L (Fig. 2B, Additional file 1: Fig. S19). The most sensitive effect on larval development was observed for the test duration of ten days with an EC_{50} of 148 ng/L (cl: 80.0–270 ng/L) and a NOEC of < 38.0 ng/L. At this observation time, 80.0% (± 16.3%) of the larvae in the control condition reached the respective larval stage L7, whereas the proportion ranged from 55.0% (± 5.8%) in the lowest concentration 38.0 ng/L to 16.7% (± 19.2%) in the highest concentration 600 ng/L (Additional file 1: Tables S5 to S7) of the exposure conditions.

After emergence and molting to the imago stage, the wing length was determined. However, no statistically significant difference in wing length was determined when comparing the treatments and the control (Additional file 1: Fig. S20). Total RNA was extracted from the female mayflies at test end in order to assess, whether the test item induced effects at the transcriptome level in emerged individuals. However, the analysis revealed no significant changes in gene expression in any exposure condition in comparison to the non-treated controls. This lack of molecular effects may have several reasons, e.g.: as soon as the mayflies had emerged, they were no longer in contact with the test item. Depending on their emergence time point and the duration until final molting from subimago to imago stage, the organisms were not exposed to the test item for up to several hours (0–24 h). Therefore, a test item-related effect at the transcriptome level may have blurred out to different extents in each individual due to the differing time periods of non-exposure, which, as well as the limited number of replicates analyzed, may have caused substantial variation leading to statistical insignificance of effects. However, considering the relatively long exposure time compared to the possible non-exposure time, this explanation can rather be assumed as unlikely. Another aspect explaining the lack of effects in gene expression may be the limited number of physiological life contents of emerged individuals, which may require a very limited repertoire of gene expression programs. In fact, transcriptional programs of adults and larvae differed by some extent. A total of 7534 genes were expressed in both adults and larvae, while 652 genes were expressed in adults only and 1940 genes were expressed in larvae only.

However, within this test we could show a concentration-dependent effect on larval development already after a short test duration. For this endpoint, a statistically significant effect was detected after seven days of exposure the earliest, indicating that sublethal effects may be detected already after a relatively short exposure time.

Table 1 Overview of EC_x and NOEC/LOEC based on nominal fipronil concentrations determined for several endpoints in the long- and short-term exposure tests with *C. dipterum*

Test	Endpoint	Nominal fipronil concentrations			
		EC_{10}	EC_{50}	NOEC	LOEC
		ng/L (95% cl)		ng/L	
Long-term exposure	Larval development after 3 days[a]	n. d	n. d	≥ 600	> 600
	Larval development after 7 days[b]	23.0[d)] (4.00–47.0)	180 (117–292)	38.0	75.0
	Larval development after 10 days[c]	n. d	148 (80.0–270)	< 38.0	≤ 38.0
	Mortality after 38 days	n. d	185 (85.0–571)	38.0	75.0
	Emergence after 38 days	n. d	160 (67.0–426)	38.0	75.0
Short-term exposure	Larval development after 3 days[a]	n. d	n. d	≥ 300	> 300
	Larval development after 7 days[b]	25.0[d] (n. d.)	n. d	75.0	150
	Mortality after 7 days	n. d	n. d	≥ 300	> 300

cl = confidence limits; n. d. = not determined due to mathematical reasons

[a] Respective larval stage L4

[b] Respective larval stage L6

[c] Respective larval stage L7

[d] Extrapolated value

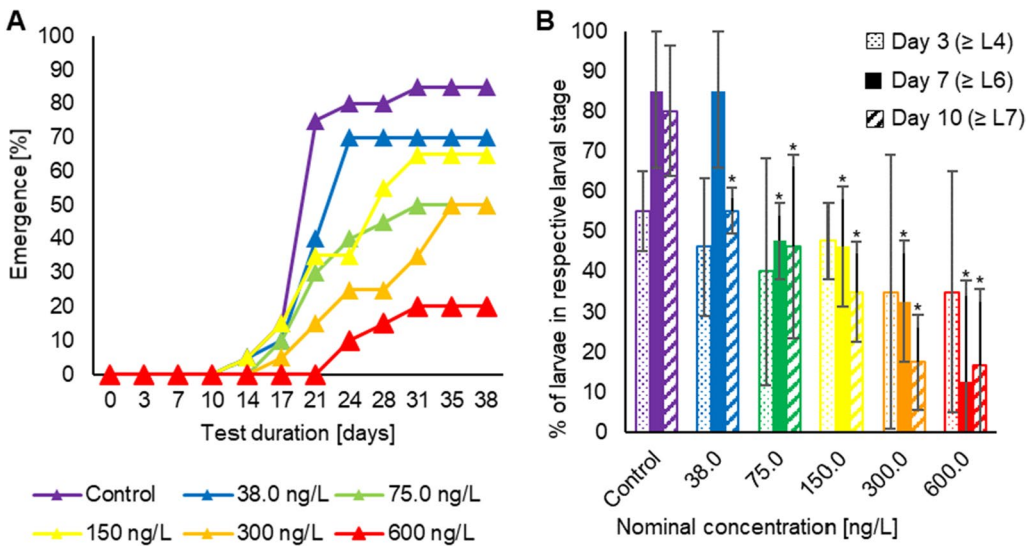

Fig. 2 **A** Emergence per treatment ($n = 20$ per treatment) in the long-term toxicity test with *C. dipterum* with a total test duration of 38 days and semi-static test design. A control and five fipronil concentrations 38.0–600 ng/L were tested. **B** Percentage of *C. dipterum* larvae in the respective larval stage at Day 3 (≥ L4), Day 7 (≥ L6) and Day 10 (≥ L7) per treatment in the long-term toxicity test. A control and five fipronil concentrations 38.0–600 ng/L were tested. A significant inhibition compared to the control is marked (*)

With increasing test duration, the sublethal effect may have been overlaid by the increasing mortality. Therefore, we developed a short-term exposure test with a total test duration of seven days covering the probably most sensitive life stages in order to include transcriptome analysis of larvae at test end.

Short-term exposure test: fipronil effects on physiological endpoints in *C. dipterum*

Chemical analysis of fipronil concentrations in the short-term exposure test revealed recoveries between 78 and 89 %, thus fipronil effects were evaluated based on nominal concentrations (Additional file 1: Table S4). In the short-term exposure test performed with a test duration of seven days, no mortality was observed in the

control group. The mortality in the fipronil exposure concentrations ranged from 10% (concentrations 38.0 and 150 ng/L) to 20% (concentrations 75.0 and 300 ng/L) (Additional file 1: Fig. S21). No concentration-related effect was revealed for the endpoint mortality by Probit analysis and the NOEC was determined to be ≥ 300 ng/L, indicating that the tested concentration range was sublethal for the test duration of seven days. Like in the long-term exposure test, no statistically significant effect on the larval development was revealed by Probit analysis after a test duration of 3 days and the NOEC was determined as ≥ 300 ng/L. For the test duration of 7 days, an EC_{10} of 25.0 ng/L (extrapolated, cl: n. d.) was determined, however, an EC_{50} could not be calculated (Table 1). The NOEC was determined as 75.0 ng/L. At this observation time, 80.0% (± 17.9%) of the larvae in the control reached the respective larval stage L6, whereas in the conditions exposed to fipronil the proportion ranged from 78.3% (± 31.4%) in the lowest concentration of 38.0 ng/L to 45.0% (± 5.8%) in the highest concentration of 300 ng/L (Additional file 1: Fig. S22, Tables S8 and S9). In comparison to the results of the long-term exposure test, the larval development in the control condition and also the effect in the treatments was reproducible after 7 days, pointing out larval development as an appropriate sublethal endpoint. In the short-term exposure test, no emergence was observed. Given these results, we expected to be able to identify differentially expressed genes as a consequence of fipronil exposure at the end of the developed short-term test.

Functionality of the test system

Until now, only few studies have investigated chronic toxicity in EPT taxa. Soucek and Dickinson [38] described a test system for chronic toxicity tests with the North American mayfly species *Neocloeon triangulifer* investigating the development rate as sublethal endpoint. Chronic toxicity of neonicotinoids to the New Zealand mayfly species *Deleatidium* spp. was tested by investigating immobility, impairment and molting propensity of the larvae [21]. However, the literature is lacking data on chronic toxicity testing with European EPT taxa. Since there are several indications that EPT taxa can be highly sensitive to various environmental pollutants as neonicotinoids, the establishment of a test system to test chronic toxicity in a European mayfly species is demanded [34]. Within the present study, two chronic toxicity tests of different durations were performed with *C. dipterum*. *C. dipterum* was shown to be a suitable test species within the EPT taxa because of its lentic living and the high abundance in regional waterbodies [4, 8]. In the present study, field-collected organisms were used, which can lead to uncertainties regarding the health state of the organisms, infection with parasites, the influence of different environmental impacts and the overall biological variety. For example, van den Brink et al. [41] have shown great differences in the susceptibility to environmental pollutants when analyzing two *C. dipterum* generations [41]. Additionally, the speed of larval development has been shown to be greatly influenced by environmental impacts [8]. In our study, we also observed that few larvae paused in their development independent from fipronil exposure. However, the possibility for the establishment of a laboratory culture makes *C. dipterum* a promising representative for EPT taxa [2].

In the long-term exposure test performed, covering nearly the whole life span of the organisms from young larvae (larval stage L3) until emergence, the control mortality was 10% and the control emergence was 85% at test end, indicating that the test conditions were suitable for *C. dipterum*. In addition, the low control mortality and the high control emergence, respectively, are in line with validity criteria of OECD test guidelines for chronic toxicity tests with other aquatic invertebrates like *D. magna* or *C. riparius* [25, 26]. The high control emergence of 85% indicates the test duration of 38 days is sufficient to cover the lifespan from a young larval stage until emergence. Because of the difference in the test duration, the long and the short-term exposure tests are not directly comparable. However, in the short-term exposure test no mortality was observed in the control condition, which is proving the functionality of the test system.

Transcriptome response to fipronil during *C. dipterum* larval development

The developed short-term test, among others, aimed at investigating the ability to include the assessment of molecular endpoints after fipronil exposure at the transcriptome level. For this, RNA-seq-based transcriptome analysis was performed. Larvae exposed to the sublethal concentrations of fipronil of 75.0, 150 and 300 ng/L at test end (day 7) were compared to the non-treated controls. Principal component analysis (PCA) of the gene expression profiles across all experimental conditions and biological replicates revealed a comparatively clear separation between treatment and the control groups, with samples from the higher exposure concentrations differing significantly more from controls than those from the lowest fipronil concentration studied. The first principal component (PC1) accounted for 32.4% of the total variation in the dataset (Fig. 3A).

This allowed for the identification of underlying patterns and gene expression differences between conditions. Out of the 9474 genes that were identified as expressed across all samples, 3 genes were found to be significantly regulated in response to a concentration

of 75.0 ng/L fipronil (low exposure), while 474 genes showed significant regulation after exposure to 150 ng/L (mid exposure), and 1137 genes were observed to be regulated after exposure to 300 ng/L (high exposure), all conditions with a high degree of overlap and positive correlation with regard to the direction of expression changes (Fig. 3B and C). Remarkably, 137 (50%) of the 276 DEGs responsive to medium and high exposure were not expressed in adult *C. dipterum*, strongly suggesting that the limited repertoire of gene expression programs in adult *C. dipterum* is responsible for their insensitivity at the molecular level.

Functional processes affected by fipronil

To better understand the processes disrupted by the GABA-gated chloride channel blocker fipronil, we performed functional annotation of the *C. dipterum* reference genome by employing homology-based gene prediction. By mapping them to a closely related genus, this approach allowed us to assign Gene Ontology (GO) terms to the corresponding *C. dipterum* (Additional file 1: Fig. S23) [19]. Subsequently, we conducted overrepresentation analysis (ORA) using the genes that showed significant regulation in response to the different treatments (Additional file 1: Fig. S24). Gene set enrichment analysis (GSEA) was also carried out on the complete expression profiles for the comparison of each treatment condition against the control to gain insight into the affected biological processes and molecular functions (Additional file 1: Fig. S25). The results of the analysis were consistent with the known MoA of fipronil, as it was found to significantly regulate gene sets involved in synaptic signaling, ion channel activity or transmembrane transport to neurotransmitter activity (Fig. 4A, B and Additional file 1). Fipronil has been extensively studied for its inhibitory effects on the γ-aminobutyric acid (GABA) receptor, resulting in the disruption of chemical synaptic transmission [37], hyperexcitability of neurons [42], neurobehavioral abnormalities [15] and impaired neurotransmission [22]. The neurotoxic effects of fipronil during the critical developmental stages of zebrafish, as well as in rat and mice models, have been consistently demonstrated in a number of in vivo studies [1, 3, 9, 27, 46]. These in vivo investigations collectively provide evidence for the neurotoxic effects of fipronil across different species.

The dysregulation of ion transport, including neurotransmitter transport, implies a disruption in the control of ion concentrations across cellular membranes [5]. Proper regulation of ion transport is crucial for maintaining optimal cell function, including the generation and propagation of action potentials, synaptic transmission, and the establishment of membrane potentials necessary for cellular communication [43]. Moreover, the disturbance in transmembrane transport as indicated by our results, particularly that involving metal ions, can have a major impact on cellular homeostasis, the preservation of appropriate ion gradients, and the general cellular health [31] of the *C. dipterum* larvae. This disruption in ion and neurotransmitter transport may have a significant impact on neuronal excitability, cellular signaling, and the overall functionality of the nervous system.

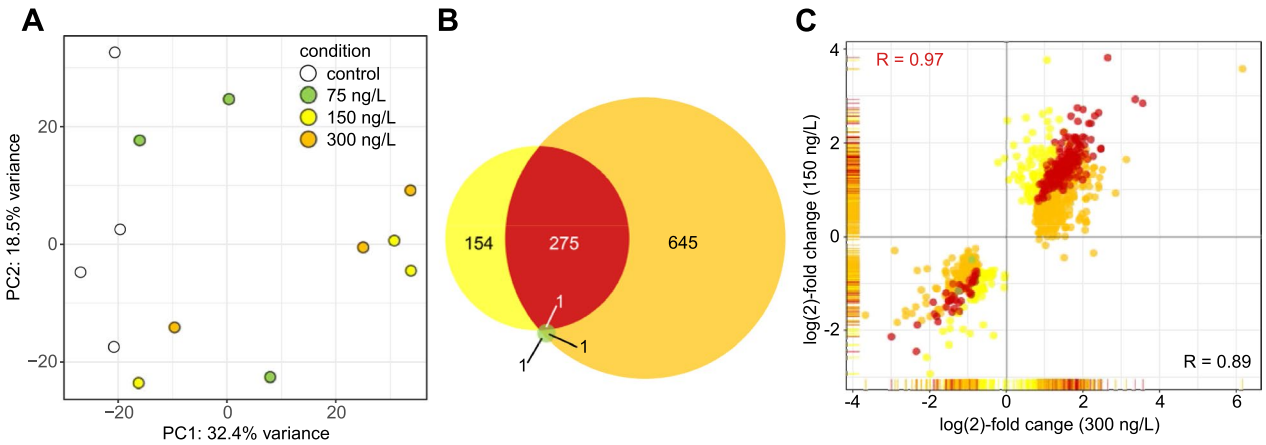

Fig. 3 Differentially expressed genes (DEGs) after exposure of *C. dipterum* larvae to fipronil. **A** Principal component analysis (PCA) of the biological replicates of treated and non-treated samples, following the exposure of *C. dipterum* larvae to low (75.0 ng/L), mid (15.0 ng/L), and high (300 ng/L) concentrations of fipronil. **B** Venn diagram depicting the number of DEGs in each exposure condition. Conditions are colored as in **A**. **C** Scatter plot comparing DEGs log(2) fold change (lfc) values after low, mid and high exposure conditions. The common subset of the middle and the high exposure is highlighted in red

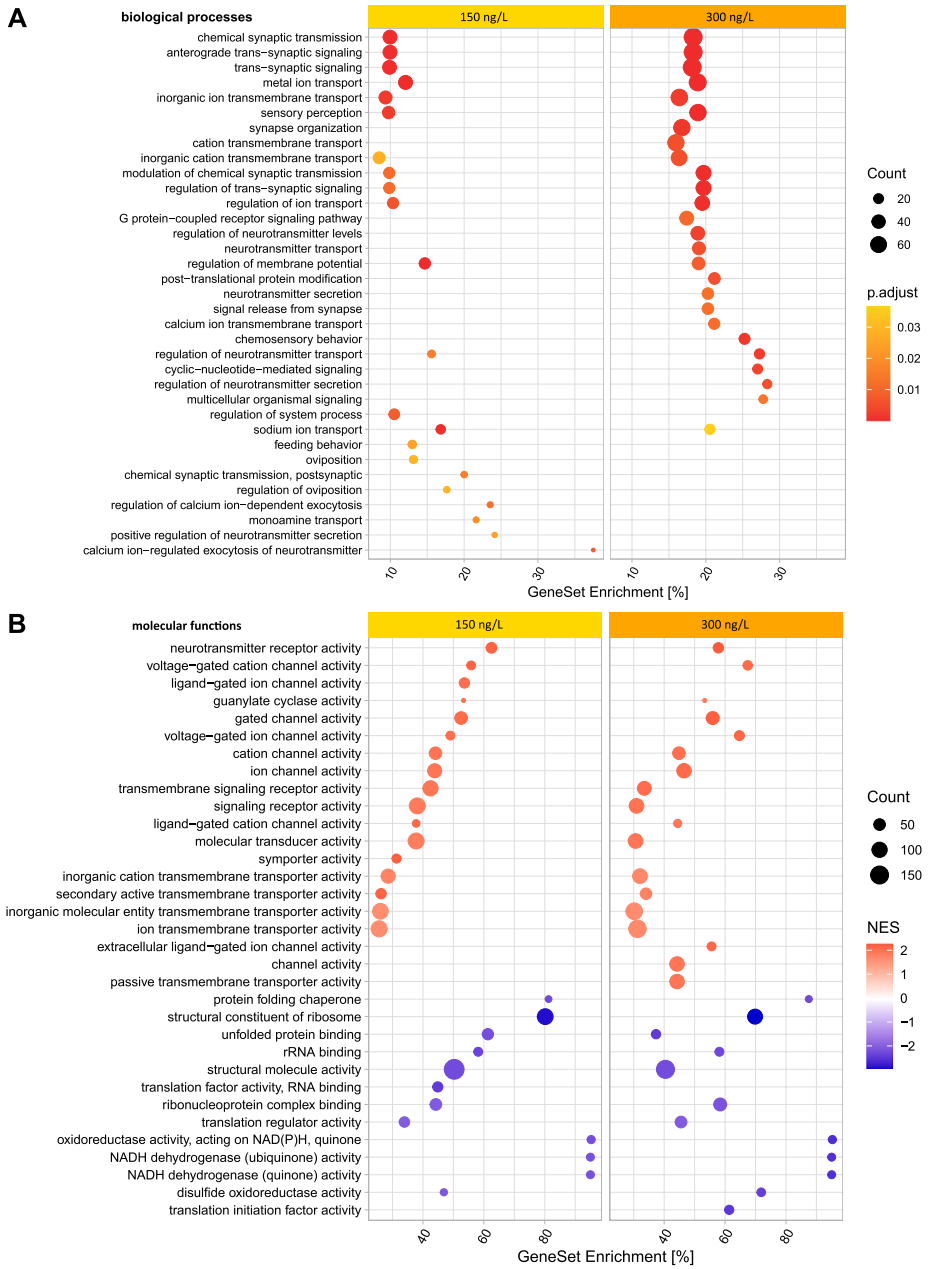

Fig. 4 Dot plot depicting the selection of significantly regulated overrepresented biological processes as obtained from ORA and enriched molecular functions as observed by GSEA after exposure of *C. dipterum* larvae to sublethal concentrations of fipronil. **A** Biological processes overrepresented and **B** molecular functions enriched in the dataset. In each plot, bubbles were drawn for the top 10 terms identified as statistically significantly overrepresented (**A**) or enriched (**B**) (with a Benjamini–Hochberg adjusted *p*-value of ≤ 0.05) for the corresponding treatment. The size of each bubble corresponds to the log(2) transformed count of genes identified as enriched in a particular gene set (count). The gene set enrichment (in percentage) determines the position of the bubble on the *x*-axis and indicates how many genes in a gene set were enriched relative to the set's size. The color of the bubbles indicates the degree of significance of the corresponding overrepresented GO term (adjusted *p*-value of ≤ 0.05), which is shown in percentage on the *x*-axis (**A**) and the direction of the normalized enrichment score (NES), with positive scores shown in red and negative scores in blue (**B**)

Taken together, the consistency of the overrepresented or enriched biological processes and molecular functions with the MoA of fipronil validates our homology-based functional annotation of the *C. dipterum* reference genome. Furthermore, these findings strongly support that the observations made at the

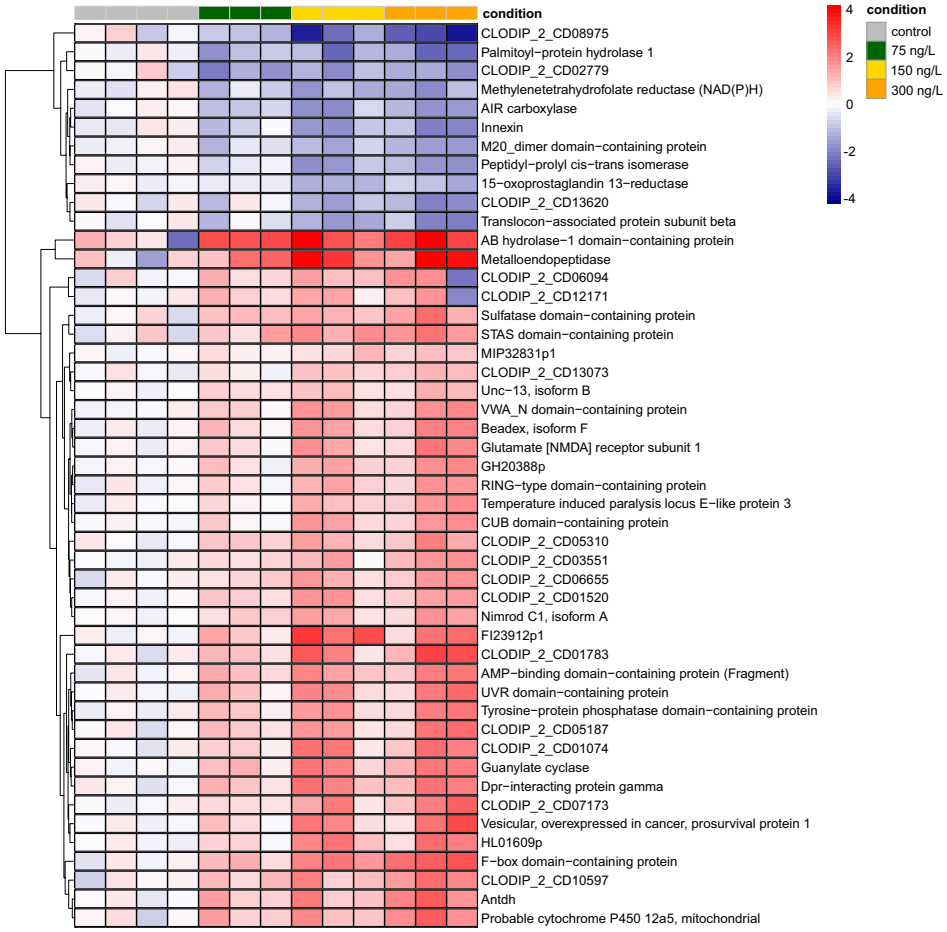

Fig. 5 Heatmap showing relative gene expression patterns of *C. dipterum* larvae post exposure to sublethal concentrations of fipronil. The top 48 core DEGs are shown based on their adjusted *p*-values (rows). The relative expression signal for each gene is shown as the variance-transformed normalized counts, centered around the control group's mean and scaled by the global standard deviation. The color red indicates an enhanced expression and the color blue denotes a suppressed expression relative to the global control's mean expression of a gene. The color above each column indicates the corresponding exposure condition. Each row stands for a biological replicate

molecular level are meaningful, which is a prerequisite for the identification of marker gene candidates.

Transcriptome marker gene candidates targeted by fipronil in *C. dipterum* larvae

As the gene sets responsive to fipronil were supported by the MoA of the substance, we focussed on the DEGs identified in the common subset of the higher fipronil exposure concentrations (150 and 300 ng/L) for the identification of suitable marker gene candidates. We applied an adjusted *p*-value sorting approach following Benjamini–Hochberg correction for multiple testing ($p < 0.05$) to these core DEGs. The resulting set of 48 top DEGs based on the significance of their regulation (as shown in Fig. 5) was then used as input for hierarchical clustering analysis to explore the expression patterns observed between the treated groups and untreated control groups. The complete list of the 171 DEGs found in the common set of mid and high exposure condition with counts per million read values (CPM) over 10 is shown in Additional file 1: Fig. S27.

Common target genes were regulated in the same direction when comparing different fipronil exposure conditions in comparison to the non-treated control (Fig. 5). Thus, this suggests that fipronil affects the expression of these genes consistently, regardless of exposure concentration. Downregulated genes include *palmitoyl-protein hydrolase 1* (CLODIP_2_CD06443), *methylenetetrahydrofolate reductase* (NAD(P)H) (CLODIP_2_CD06421), *peptidyl-prolyl cis–trans isomerase* (CLODIP_2_CD13620) and the *translocon-associated protein subunit beta* (CLODIP_2_CD14265). *Palmitoyl-protein hydrolase 1* is a member of the thioesterase superfamily involved in lipid metabolism, protein modification and signal transduction. A previous study has connected the activation of unfolded protein response (UPR), neuronal apoptosis and degeneration in mice to palmitoyl-protein

hydrolase 1 deficiency [47]. *Methylenetetrahydrofolate reductase (MTHFR)* plays a critical role in folate metabolism and catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. *MTHFR* gene deficiency has been previously associated with neuronal defects and developmental disorders in zebrafish [36]. *Peptidyl-prolyl cis–trans isomerase* (PPIase) is a member of the larger enzyme class known as isomerases, which have profound impact on key proteins involved in the regulation of cell growth, immune-related disorders and neuronal differentiation. Shortfalls in the *peptidyl-prolyl cis–trans isomerase* have been previously associated with apoptosis and misfolded proteins, which are the hallmarks of several neurodegenerative disorders [40].

On the other hand, genes such as *metalloendopeptidase* (CLODIP_2_CD03171), *beadex, isoform F* (CLODIP_2_CD06105) and the *glutamate receptor subunit 1* (CLODIP_2_CD14203) were significantly upregulated after fipronil exposure. *Metalloendopeptidase* is an enzyme belonging to the class of endopeptidases, which play an essential role in a variety of physiological processes, including protein degradation, peptide hormone processing and regulation of the immune system. The involvement of *metalloendopeptidase* enzymes in the degradation of gonadotropin-releasing hormone (GnRH) has been demonstrated in a previous study [14]. GnRH is a neuropeptide hormone that plays an essential role in the brain during embryonic development [33, 45]. The Beadex (Bx) gene plays a critical role in the motor neurons, which are essential for the proper functioning of the reproductive system in female flies [18]. *Glutamate receptor subunit 1* (*GRIN1*) is a member of the glutamate receptor channel superfamily involved in mediating excitatory neurotransmission and synaptic plasticity in the central nervous system (CNS). As a subunit of the N-methyl-D-aspartate receptor, *GRIN1* plays a critical role in several physiological processes and neuronal functions. Previously, a spectrum of developmental delays, ranging from mild to profound, has been associated with *GRIN1*-related neurodevelopmental disorders [28]. Taken together, the observed gene expression changes identified in this study are in line with existing scientific literature on neurodevelopmental disorders and therefore may represent promising marker gene candidates for the neurotoxic MoA of fipronil in *C. dipterum* as a model organism.

Conclusion

In this study, we were able to identify a suitable test protocol for chronic toxicity testing with *C. dipterum* from a young larval stage until emergence including sensitive sublethal endpoints, such as larval development and emergence, for application in SSD approaches. Within a long-term exposure test, we found a high sensitivity of *C. dipterum* to fipronil.

Based on the results of this long-term exposure test, we developed a test with a shorter test duration, reproducing the observed effects on larval development and allowing the inclusion of a transcriptome-based assessment of molecular endpoints. For this, we functionally annotated the *C. dipterum* reference genome using a homology-based approach. Transcriptomics revealed significant concentration-related effects at the molecular level, which were in line with the MoA of the test substance and allowed the identification of marker gene candidates. Thus, we were able to show sensitive sublethal effects already after a 7 days exposure by implementation functional transcriptome analysis. This shortened assay allows for early identification of toxicity of chemicals in *C. dipterum* in a MoA-differentiating manner, thereby providing the possibility of assessing chronic toxicity in the challenging non-standard organism *C. dipterum* as representative for EPT taxa. However, further testing using this methodology is required in the future in order to validate the procedure.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12302-023-00806-4.

Additional file 1: Table S1. Composition of nutrient solution for *Navicula pelliculosa* culture. **Table S2.** Water parameters of the long- and short-term exposure tests. **Table S3.** Time-weighted mean concentrations of fipronil of the long-term exposure test. **Table S4.** Time-weighted mean concentrations of fipronil of the short-term exposure test. **Table S5.** Percentage of larvae ≥ L4 per replicate and treatment after three days exposure in the long-term exposure toxicity test with *C. dipterum*. **Table S6.** Percentage of larvae ≥ L6 per replicate and treatment after seven days exposure in the long-term exposure toxicity test with *C. dipterum*. **Table S7.** Percentage of larvae ≥ L7 per replicate and treatment after ten days exposure in the long-term exposure toxicity test with *C. dipterum*. **Table S8.** Percentage of larvae ≥ L4 per replicate and treatment after three days exposure in the short-term exposure toxicity test with *C. dipterum*. **Table S9.** Percentage of larvae ≥ L6 per replicate and treatment after seven days exposure in the short-term exposure toxicity test with *C. dipterum*. **Figure S1.** Left. *C. dipterum* larva in larval stage L3; Right. *C. dipterum* larva in larval stage N1. **Figure S2.** Larval development stages based on wing pad development of *C. dipterum* (adopted from Cianciara 1976). **Figure S3.** Molecular sturcture of the test substance fipronil. **Figure S4.** RNA-Seq read count normalization using DESeq2. (A) Raw read counts (left) and relative log expression (RLE) normalized read counts (right) of adult *C. dipterum* exposed to low (75.0 nµg/L) and high (300 ng/L) concentrations of fipronil and corresponding controls. Biological replicates are numbered as shown. **Figure S5.** Distributions of p-values, p-value conversion, and distributions of log(2)-fold changes upon low and high levels of fipronil exposure in adult *C. dipterum* observed by gene expression data in contrast to the control. Distribution of p-values for all genes after exposure to (A) low (75.0 n/L) and (B) high (300 ng/L) fipronil concentrations (Wald's t-test) (top). Wald's p-values were obtained and compared to converted p-values for multiple testing using Benjamini–Hochberg, as well as the corresponding p-values for exposure to low (75 ng/L) and high (300 ng/L) concentrations of fipronil (Middle). The distribution of log(2)-fold change values for all genes following fipronil exposure at low (75.0 ng/L) and high (300 ng/L) concentrations (bottom).

For each case, the dotted line denotes the log(2)-fold change cut-off. **Figure S6.** MA-plot (top) showing log(2) transformed mean expression (gene count) to apeglm shrunk log(2)-fold change for every expressed gene in (A) Low and (B) High exposure condition in comparison to the control and Volcano plot (bottom) depicts the log(2)-fold change (lfc) values against the corresponding –log(10)(padj) values of genes that were differentially expressed after (A) Low and (B) High fipronil exposure to adult *C. dipterum*. The lfc value cut-off as well as the padj cut-off are indicated as dotted lines. Genes applying to both of them are colored red. Black dots represent genes with no significant difference. **Figure S7.** PCA (A) and t-distributed stochastic neighbor embedding (t-SNE) (B) of adult *C. dipterum* exposure to low (75.0 ng/L) and high (300 ng/L) concentrations of fipronil, as showed by RNA-Seq. On top of each panel, the total number of normalized genes used for clustering is displayed. **Figure S8.** Sample distance matrix analysis of adult *C. dipterum* exposed to fipronil at low (75.0 ng/L) and high (300 ng/L) concentrations, as well as the corresponding controls. The conditions are denoted by a color code. **Figure S9.** RNA-Seq read count normalization using DESeq2. (A) Raw reads (left) and Relative log Expression (RLE) normalized read counts (right) of *C. dipterum* larvae exposed to low (75.9 ng/L), mid (1500 ng/L), and high (300 ng/L) concentrations of fipronil, as well as the corresponding controls. Biological replicates are numbered. **Figure S10.** Distributions of p-values, p-value conversion, and distributions of log(2)-fold changes following low, mid, and high exposure of *C. dipterum* larvae to fipronil as observed by gene expression data in comparison to the control. Distribution of p-values for all genes following exposure to (A) low (75.0 ng/L), (B) mid (150 ng/L), and (C) high (300 ng/L) concentrations of fipronil (Wald's t-test) (top). Obtained Wald's p-values in comparison to converted p-values for multiple testing following Benjamini–Hochberg, as well as the corresponding p-values for exposure to low (75.0 ng/L), mid (150 ng/L), and high (300 ng/L) concentrations of fipronil (Middle). Distribution of log(2)-fold change values of all genes after exposure to fipronil at low (75.0 ng/L), mid (150 ng/L), and high (300 ng/L) concentrations (bottom). The dotted line represents the log(2)-fold change cut-off for each condition. **Figure S11.** MA-plot (top) showing log(2) transformed mean expression (gene count) to apeglm shrunk log(2)-fold change for every expressed gene in (A) Low, (B) Mid and (C) High exposure condition in comparison to the control and Volcano plot (bottom) depicts the log(2)-fold change (lfc) values against the corresponding –log(10)(padj) values of genes that were differentially expressed after (A) Low, (B) Mid and (C) High fipronil exposure to *C. dipterum* larvae. The lfc value cut-off as well as the padj cut-off are indicated as dotted lines. Genes applying to both of them are colored red. Black dots represent genes with no significant difference. **Figure S12.** PCA (A) and t-distributed stochastic neighbor embedding (t-SNE) (B) of samples upon exposure to low (75.0 ng/L), mid (150 ng/L), and high (300 ng/L) concentrations of fipronil to *C. dipterum* larvae as showed by RNA-Seq. On top of each panel, the total number of normalized genes used for clustering is displayed. **Figure S13.** Sample distance matrix analysis of *C. dipterum* larvae samples exposed to fipronil at low (75.0 ng/L), mid (150 ng/L), and high (300 ng/L) concentrations, as well as the corresponding controls. The conditions are denoted by a color code. **Figure S14.** Chromatogram of fipronil (quantifier and qualifier mass transition) of the control sample at day 21 (fresh) of the chronic test. The measured concentration in this sample was 0.018 µg/L (<LOQ). The y-axis was scaled to the conc. 1 sample at the same day (see Figure S 4). **Figure S15.** Chromatogram of fipronil (quantifier and qualifier mass transition) of the concentration level 1 sample at day 21 (fresh) of the chronic test. The measured concentration in this sample was 0.052 µg/L. **Figure S16.** Chromatogram of fipronil (quantifier and qualifier mass transition) of the control sample at day 21 (fresh) of the short-term exposure test. No peak was detected (n.d.). The y-axis was scaled to the conc. 1 sample at the same day (see Figure S 6). **Figure S17.** Chromatogram of fipronil (quantifier and qualifier mass transition) of the concentration level 1 sample at day 21 (fresh) of the short-term exposure test. The measured concentration in this sample was 0.027 µg/L. **Figure S18.** Mortality induced by fipronil in *C. dipterum* per treatment during a long-term exposure test with a total duration of 38 days. **Figure S19.** Larval development in each treatment for the test duration of 38 days in the long-term exposure test. **Figure S20.** Wing size of *C. dipterum* imagines per treatment during in the long-term exposure test with a total duration of 38 days. **Figure S21.** Mortality induced by fipronil in *C. dipterum* per treatment during the short-term exposure test with a total duration of 7 days. **Figure S22.** Larval development in each treatment for the test duration of 7 days in the short-term exposure test. **Figure S23.** Total blastp top hit search results per genus illustrating the top hits distribution in relation to the C. dipterum gene IDs from the reference genome. **Figure S24.** Overrepresentation analysis of significantly regulated genes (A) Biological processes (B) Molecular functions following the sublethal exposure exposure of *C. dipterum* larvae to fipronil. The log(2)-converted gene count for each ontology term is represented by the size of the bubble, and the geneset enrichment (%) is plotted on the x-axis. For biological processes that are statistically significantly enriched (BH adjusted p 0.05), bubbles are drawn. Color codes are assigned to adjusted p-values. **Figure S25.** Gene set enrichment analysis of all identified genes (A) Biological processes (B) Molecular functions following sublethal exposure of *C. dipterum* larvae to fipronil. The size of the bubble represents the log(2)-converted gene count for each ontology term, and the x-axis plots the geneset enrichment (%). The colors of the bubbles represent a positive (red) or negative (blue) normalized enrichment score (NES). Geneset enrichment is a percentage measure of how many genes in a given gene set were enriched relative to set size (GeneSet = (Count / gene set size) * 100). **Figure S26.** Gene network cluster showing the top 20, most significantly (padj BH $\leq$ 0.05) enriched (A) Biological processes (B) Molecular functions GO terms from gene set enrichment analysis (GSEA). As input for the GSEA, substance specific gene sets were selected based on the shared DEGs between mid (150 ng/L) and high (300 ng/L) exposure condition. GO term bubble size reflects the number of genes from the input gene set associated with this biological process. Red indicates an enhanced and blue a suppressed expression relative to the global mean expression of a gene linked with the corresponding GO terms. **Figure S27.** Heatmap illustrating the relative expression patterns of the common set of ME and HE DEGs ranked by their abs(lfc) for genes above 10 CPM (counts per million reads). Relative to the global control mean expression of a gene, red indicates upregulation and blue indicates downregulation. Gene clustering was performed using Euclidean distance. The color code above each column describes the assignment of the sample to the respective exposure condition.

Additional file 2. Full ORA results as obtained from clusterProfiler analysis.

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Author contributions
K.G. performed experiments and data analysis for physiological endpoints and wrote the manuscript. S.U.A. contributed to analysis of transcriptome data and wrote the manuscript. H.R. analyzed the transcriptome data and developed the functional annotation pipeline. M.V. contributed to study design and supervision of the study. B.R. and B.G. performed chemical analysis. E.E. contributed to data analysis and manuscript writing. L.K. and S.E. contributed to study design and supervision of the study. All authors reviewed the manuscript.

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Availability of data and materials
The data generated or analyzed during this study are included in this published article and Additional files 1, 2. Transcriptome datasets generated and analyzed during the current study are available in the ArrayExpress database at EMBL-EBI (www.ebi.uk/arrayexpress) under accession numbers E-MTAB-12814. The org.Cdipterum.eg.db package and the corresponding data

files used to create this annotation are publicly available on Zenodo (www.zenodo.org) under accession number 8003805 [29]. Further figures, tables, detailed methodological descriptions as well as ORA results can be found in Additional files 1, 2.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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