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Research Paper

Environmental levels of carbaryl impair zebrafish larvae behaviour: The potential role of ADRA2B and HTR2B

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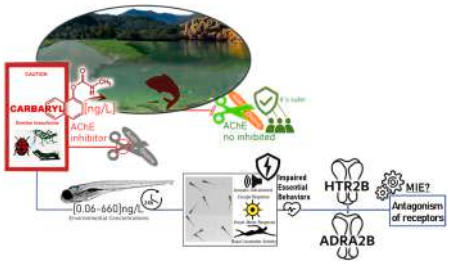
HIGHLIGHTS

- Environmental levels of carbaryl impair zebrafish larvae behavior.
- Effect of carbaryl on behavior is acetylcholinesterase-independent.
- ADRA2B and HTR2B are molecular targets for carbaryl validated in *in vitro* assays.
- Similar effects on larvae behavior with carbaryl and antagonists of ADRA2B and HTR2B.
- Environmental levels of carbaryl are unsafe for fish communities.

GRAPHICAL ABSTRACT



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ABSTRACT

The insecticide carbaryl is commonly found in indirectly exposed freshwater ecosystems at low concentrations considered safe for fish communities. In this study, we showed that after only 24 h of exposure to environmental concentrations of carbaryl (0.066–660 ng/L), zebrafish larvae exhibit impairments in essential behaviours. Interestingly, the observed behavioural effects induced by carbaryl were acetylcholinesterase-independent. To elucidate the molecular initiating event that resulted in the observed behavioural effects, *in silico* predictions were followed by *in vitro* validation. We identified two target proteins that potentially interacted with carbaryl,

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; ADRA2B, α2B adrenoceptor; ADRB1, β1 adrenoceptors;; BLA, basal locomotor activity; BPM, beats per minute; dpf, days post-fertilization; ERA, environmental risk assessment; fps, frames per second; HR, heart rate; HTR2B, serotonin 2B receptor; LLC, long latency C-bend; MAO, monoamine oxidase; MIE, molecular initiating event; MoA, mode of action; NMCR, nonmonotonic concentration–response; PCPA, p-chlorophenylalanine; SERT, serotonin transporter; SLC, short latency C-bend; TPH, tryptophan hydroxylase; VMR, visual motor response; VSR, vibrational startle response or startle.

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ADRA2B
HTR2B

the α2B adrenoceptor (ADRA2B) and the serotonin 2B receptor (HTR2B). Using a pharmacological approach, we then tested the hypothesis that carbaryl had antagonistic interactions with both receptors. Similar to yohimbine and SB204741, which are prototypic antagonists of ADRA2B and HTR2B, respectively, carbaryl increased the heart rate of zebrafish larvae. When we compared the behavioural effects of a 24-h exposure to these pharmacological antagonists with those of carbaryl, a high degree of similarity was found. These results strongly suggest that antagonism of both ADRA2B and HTR2B is the molecular initiating event that leads to adverse outcomes in zebrafish larvae that have undergone 24 h of exposure to environmentally relevant levels of carbaryl.

1. Introduction

Carbaryl (1-naphthyl-N-methylcarbamate) is the active ingredient of Sevin™, one of the most widely used carbamates for the control of a broad spectrum of insects and a general garden insecticide (Hastings et al., 2001; Gunasekara et al., 2008a). Carbaryl is moderately soluble in water (≈40 mg/L) (Hastings et al., 2001), and while its half-life in laboratory conditions is only a few days, studies have reported that it can persist for months in some aquatic ecosystems (Gibbs et al., 1984; Liu et al., 1981). As a result, carbaryl residues have been detected in surface waters adjacent to both agricultural and urban areas, commonly at ng/L concentrations. For example, environmental levels of carbaryl in some freshwater ecosystems are as follows: 330–1300 ng/L in surface water at Ten Mile Creek (US, 2001–2002) (Wilson and Foos, 2006); 10–100 ng/L and 337 ng/L in the Pinus and Strymonas rivers, respectively (Greece, 2013) (Fytianos et al., 2006; Terzopoulou et al., 2015); 44–1865 ng/L in the Ebro River basin (Spain, 2010–2011) (Herrero-Hernández et al., 2017); and 2–7 ng/L in rivers entering Corner Inlet Marine National Park (Australia, 2009–2010) (Allinson et al., 2016). Higher concentrations, measured in µg/L to mg/L, have been reported in areas directly impacted by the use of carbaryl to control insect pests (Walters et al., 2003; Labenia et al., 2007; Derbalah et al., 2020).

Carbaryl is a neurotoxic compound whose main mode of action (MoA) is the inhibition of the enzyme acetylcholinesterase (AChE), which is responsible for converting the neurotransmitter acetylcholine (ACh) into acetate and choline at cholinergic synapses. In addition to killing insect pests, carbaryl may exhibit toxic effects in nontarget species, specifically aquatic organisms. Carbaryl is toxic to water fleas, shrimp, and freshwater snails at concentrations measured in µg/L (Gunasekara et al., 2008a) and toxic to fish concentrations measured in mg/L (Gunasekara et al., 2008a, 2008b). AChE inhibition is closely associated with reduced swimming and feeding behaviours in fish. Furthermore, exposure to carbaryl at concentrations as low as 10 µg/L significantly increases the vulnerability of fish to predation (Little and Finger, 1990).

The zebrafish (*Danio rerio*) is one of the most common model fish species in ecotoxicology (Faria et al., 2020; Garcia-Reyero et al., 2014). This vertebrate model species is increasingly used in biomedical research, including neurotoxicology studies (Faria et al., 2015; Tingaud-Sequeira et al., 2017; Prats et al., 2017), since the general organization of its nervous system and its neurotransmitter systems are highly similar to humans. Recently, zebrafish larvae were used to screen for environmental contaminants with adverse effects on the vibrational startle response (Faria et al., 2020). Carbaryl was among these contaminants, emphasizing the need for further characterization of the potential adverse neurobehavioural effects of this insecticide on larval fish communities.

In this study, we first analysed the behavioural effects of a 24 h exposure to environmentally relevant concentrations (0.066–660 ng/L) of carbaryl in zebrafish larvae at 7 days post-fertilization (dpf). We demonstrated that neither AChE inhibition nor the activation of the androgenic/antiandrogenic pathway are key to the adverse behavioural effects observed in exposed larvae. Therefore, to understand the mechanisms leading to these adverse effects, we then explored the potential molecular targets of carbaryl by *in silico* molecular profiling followed by *in vitro* validation of the most relevant predictions. The predicted molecular targets that were confirmed by the *in vitro* validation were further validated in zebrafish larvae using a pharmacological approach. Finally, to better understand the involvement of the monoaminergic system in the observed behavioural effects, we evaluated the monoaminergic neurochemical profile and the expression of several genes involved in transport, metabolism and receptors.

2. Materials and methods

2.1. Fish husbandry and larvae production

Embryos and larvae from wild-type zebrafish were obtained at the CID-CSIC facilities following standard protocols (see Supplementary Methods for additional details). All the research was conducted according to the institutional guidelines (licence n° 9027).

2.2. Waterborne exposure conditions and analyses of actual concentrations and stability

Carbaryl (Pestanal®, purity 99.94%) was purchased from Sigma–Aldrich (Steinheim, Germany). Five nominal concentrations of carbaryl were tested, ranging from 0.06 to 600 ng/L. The actual concentrations of carbaryl in the fish water [reverse-osmosis purified water containing 90 µg/mL of Instant Ocean (Aquarium Systems, Sarrebourg, France) and 0.58 mM $CaSO_4{\cdot}2H_2O$] samples were determined by ultrahigh-performance liquid chromatography with triple quadrupole detector (UPLC–MS/MS) analysis (more details are provided in the Supplementary Methods). Moreover, the stability of carbaryl in the fish water during the exposure period (24 h) was estimated for all the tested concentrations by analysing the carbaryl content (UPLC–MS/MS) in water samples maintained at the same environmental conditions as that containing the zebrafish larvae (28.5 °C and 12 L:12 D photoperiod) at time points 0 and 24 h.

2.3. Carbaryl exposures

The carbaryl concentrations selected for this study, 0.066 ng/L, 0.66 ng/L, 6.6 ng/L, 66 ng/L and 660 ng/L, cover the lower-middle range of the levels reported in indirectly impacted aquatic ecosystems (Wilson and Foos, 2006; Fytianos et al., 2006; Derbalah et al., 2020). Stock concentrations of carbaryl were prepared in dimethyl sulfoxide (DMSO), and the final DMSO concentration in the working solutions across all treatments was 0.1%. DMSO is the solvent usually selected for screening libraries of small molecules in zebrafish embryos or larvae, including screenings based on behavioural assessment (Vliet et al., 2017; Maes et al., 2012; Dach et al., 2019). Moreover, concentrations of 0.1% DMSO have not been reported to affect the visual motor response of zebrafish larvae (Christou et al., 2020). In a preliminary study we performed before commencing the carbaryl exposures, no differences were found in the vibrational startle response in 8 dpf zebrafish larvae exposed to control (7.02 ± 0.2 mm, n = 83) and solvent control (0.1% DMSO; 6.52 ± 0.16 mm, n = 84) solutions (Student's t test, $P = 0.4805$). No effects on systemic toxicity were found at any of the carbaryl concentrations tested.

Experiments were carried out in 48-well microplates with 1 larva/well in 1 mL of working solution. Larval behaviour was checked

immediately after 24 h of exposure (from 7 to 8 dpf), after which larvae were sampled for the different assays. All exposures were performed under the same conditions as stated in Section 2.2. For each assay, samples were collected from 2 to 3 separate experiments.

2.4. Behavioural assessments

The vibrational startle response (VSR), visual motor response (VMR) and basal locomotor activity (BLA) of zebrafish larvae were analysed as previously described (Faria et al., 2020, 2015, 2021). Briefly, 8 dpf zebrafish larvae were video-tracked in a DanioVision unit controlled by EthoVision XT13 software (Noldus, Wageningen, the Netherlands; see Supplementary Methods for additional details).

Kinematic analysis of the acoustic/vibrational escape response was performed as described elsewhere (see Supplementary Methods for additional details). Briefly, the stimulus was provided by a mini-shaker controlled by *LarvaCam* software (https://github.com/jmporta/LarvaCam), and the responses of the larvae evoked by these vibrational stimuli were recorded with a Photron Fastcam Mini UX100 high-speed camera. Finally, the kinematic parameters of the response were analysed in the *LarvaTrack* software (https://github.com/jmporta/LarvaTrack) (Faria et al., 2021).

2.5. Predation analysis

Predation analysis was conducted according to Bhattacharyya et al. with slight adaptations (Bhattacharyya et al., 2019). Dragonfly nymphs (*Sympetrum vicinum*) purchased from Carolina Biological Supply Company (Burlington, NC) were kept at room temperature (22 °C) in individual tanks with fish water that was renewed once a week.

Fifteen dragonfly nymphs similar in size were chosen (five for each treatment). A single dragonfly nymph was selected for each experiment, placed into a glass container containing 50 mL of room temperature (25 °C) water (with a 1.5-cm water column height) and allowed to acclimate for 1 h. During this period, exposed and control zebrafish larvae were also allowed to acclimate for 1 h at room temperature. Following acclimation, 10 larvae were introduced into each glass container. Each treatment had 5 replicates; therefore, a total of 50 zebrafish larvae were used per treatment. After 1 h, the number of surviving larvae was recorded.

2.6. Cardiac activity

To assess cardiac function in the zebrafish larvae, control and carbaryl-treated larvae were anaesthetized with MS222 (tricaine, 170 mg/L) and immobilized in a Petri dish in 4% methylcellulose to provide a ventral or lateral view. Then, the cardiac activity of each larva was video recorded (AVI format at 30 fps; Supplementary Video S1) with a GigE camera (UI-5240CP-NIR-GL, Imaging Development Systems, Germany) mounted onto a Motic SMZ-171 stereomicroscope. uEye Cockpit software (version 4.90; Imaging Development Systems, Germany) controlled the camera. The duration of each trial was 30 s. Videos were then analysed with a developed MATLAB script (version R2010b, MathWorks, USA). First, the tracking area was selected over the heart location in the program interface. Then, heart rate scores were automatically calculated using a custom algorithm that relates pixel intensity changes between each video frame to the heart muscle movement. Finally, data were pooled to obtain the average beats per minute (bpm) for the control and carbaryl-treated zebrafish larvae. Additional details on the MATLAB script used for the video analysis are available in the Supplementary Methods and Supplementary Fig. S1.

Supplementary material related to this article can be found online at doi:10.1016/j.jhazmat.2022.128563.

2.7. Computational analyses

Known experimental *in vitro* affinities for carbaryl were extracted from the ToxCast repository (Williams et al., 2017). Additional targets for carbaryl were predicted using CLARITY (Chemotargets CLARITY v5, 2021). The CLARITY platform uses the following ligand-based approaches that rely on descriptor-based molecular similarity: an implementation of the similarity ensemble approach, fuzzy fragment-based mapping, quantitative structure-activity relationships, a set of machine learning methods and target cross-pharmacology indices (Gregori-Puigjané and Mestres, 2006; Vidal et al., 2011). The training set for the 4799 protein target models is generated from *in vitro* affinity data available in both public and patent sources (Sharma et al., 2016). For each target prediction, the projected affinity and mode of action are provided along with a confidence score based on the number and type of methods that independently contributed to the prediction.

2.8. In vitro validation

Once the list of predicted interactions with human proteins most relevant to carbaryl safety was obtained, they were tested with *in vitro* assays. For off-target validation purposes, *in vitro* affinity testing was performed on human proteins or (when not available) on close rodent orthologues. Testing of carbaryl *in vitro* affinity to zebrafish orthologues was not attempted since these tests are not commercially available for the proteins of interest in this study. Although the translatability of interspecies data is by no means guaranteed (Cassar et al., 2020), human and rodent *in vitro* affinities are commonly used to interrogate the toxic mechanisms of small molecules in zebrafish (Griffin et al., 2017). Binding assays [glutamate (kainate) rat ion channel (GRIK) (Vidal et al., 2011), glutamate (AMPA) rat ion channel (GRIA) (Murphy et al., 1987), androgen receptor (AR) (ZAVA et al., 1979), and human oestrogen receptor (ERβ)] (Obourn et al., 1993), the Nav1.2 human sodium ion channel (SCN2A) cell based automated patch clamp assay (Supplementary Methods) and the acetylcholinesterase human enzymatic (Ellman et al., 1961) assays were carried out by Eurofins Discovery Services (Cerep, Celle l'Evescault, France; IonChannelProfiler, St. Charles, MO, USA; and Panlabs, Taiwan, Taipei). Binding of carbaryl at the human adenosine A1 receptor (ADORA1) (Klotz et al., 1997), serotonin 2B receptor (HTR2B) (Wainscott et al., 1997) and α2B adrenoceptor (ADRA2B) (Neylon and Summers, 1985) was determined by the Inno-Pharma Drug Screening and Pharmacogenomics Platform (Santiago de Compostela, Spain).

Finally, *in vitro* functional assays to evaluate beta-1 adrenoceptor activity were carried out using a HEK-293 cell line stably expressing the Epac-S^{H188} cAMP FRET biosensor (kindly provided by Karen Martinez, University of Copenhagen) (Klarenbeek et al., 2015) and human SNAP-β1AR (from Cisbio Bioassays), whose expression can be induced by using doxycycline. Cells were maintained in DMEM supplemented with 10% buffer, 100 μg/mL hygromycin B, 15 μg/mL blasticidin and 500 μg/mL G481. Twenty-four hours before the assay, all antibiotics were removed, and the cells were treated with 10 ng/mL doxycycline. All assays were performed at room temperature following a previously described protocol (Duran-Corbera et al., 2020). Briefly, DMEM was replaced by cAMP EPAC sensor buffer (14 mM NaCl, 50 nM KCl, 10 nM $MgCl_2$, 10 nM $CaCl_2$, 1 mM N-(2-hydroxyethyl)piperazine-N-ethanesulfonic acid (HEPES), and 1.82 mg/mL glucose, at a pH of 7.2), and the ligands were added at the specified concentration. The plate was then incubated for 30 more minutes, and fluorescence was measured using a Tecan Spark M20 multimode microplate reader programmed with the following wavelength settings: excitation filter 430/20 nm; emission filters 485/20 nm and 535/25 nm. FRET ratios were calculated as the ratio of the donor emission (tdcp173 V, 485 nm) divided by the acceptor emission (mTurq2Δ, 535 nm). The obtained ratios were normalized to the effect of the agonist cimaterol. Three independent experiments were performed for each concentration.

2.9. Pharmacological approach

Yohimbine (Y3525, Sigma–Aldrich) and SB204741 (1372, Tocris) were used as ADRA2B and HTR2B antagonists, respectively. A stock solution of yohimbine was prepared in DMSO, and working solutions (0.1, 1.0 and 10 μM) were prepared by diluting the stock in water, a final DMSO concentration of 0.1%. Working solutions of SB204741 (0.1 and 1 μM) were prepared directly in water.

A pharmacological approach was used to test the hypothesis that ADRA2B and HTR2B antagonism were the molecular initiating event that led to the observed adverse effects of carbaryl. Therefore, zebrafish larvae at 7 dpf were exposed for 24 h to the selected concentrations of yohimbine or SB204741, and their effects on larvae heart rate and behaviour were determined.

2.10. Monoamine oxidase activity in zebrafish larvae

Zebrafish monoamine oxidase activity was determined as described by Faria et al. (2021a). Briefly, pools of 20 larvae were homogenized and centrifuged, and monoamine oxidase (MAO) activity was immediately determined in the carefully collected supernatant using a peroxidase-linked spectrophotometric assay (see Supplementary Methods for additional details).

2.11. Acetylcholinesterase activity in individual zebrafish larvae

Zebrafish acetylcholinesterase (AChE) activity was determined as described by Faria et al. (2015). Briefly, individual larvae were homogenized and centrifuged. The S9 fraction was then collected, and 2 mM acetylthiocholine and 0.33 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were added. The product formed from the reaction between thiocholine and DTNB ions was monitored at 405 nm for 15 min (see Supplementary Methods for additional details).

2.12. Real-time qPCR

Changes in the expression of the most relevant genes in the monoaminergic and androgenic systems were confirmed *via* qRT–PCR and further analysis of the obtained data using the ΔΔCp method (Pfaffl, 2001). Supplementary Table ST1 reports the sequences of primers for the selected genes (*adra2b, cyp19a1b, cyp2k22, htr2b, mao, slc18a2, slc6a4, sult2st3, tph1a,* and *tph1b*).

2.13. Analysis of monoaminergic neurochemicals by LC–MS/MS

Monoaminergic neurotransmitters in pools of five zebrafish larvae and their corresponding deuterated internal standards were analysed by liquid chromatography coupled to a triple quadrupole detector (LC–MS/MS), essentially as described elsewhere (Gómez-Canela et al., 2018; Mayol-Cabré et al., 2020). Separation was performed with an HILIC column, and a binary mixture based on acetonitrile (A) and water (B) was employed as the mobile phase. Neurotransmitters were measured under positive electrospray ionization (ESI+), and the MS conditions of each neurochemical were optimized (including cone voltage and collision energy). Quantification was finally conducted by the internal standard methodology (see Supplementary Methods for additional details). Quality parameters are reported in Supplementary Table ST2.

2.14. Statistical analysis

Data were analysed with IBM SPSS v25 (Statistical Package 2010, Chicago, IL). To assess the normality of the data, we used Kolmogorov–Smirnov and Shapiro–Wilk tests. The Pearson chi-square test was used to analyse the effect of the startle vibrational response on larvae survival. To determine the significance of the differences between the distributions of the treatment and control groups (*i.e.*, normal or non-normal distributions), we used a one-way ANOVA followed by Dunnett's multiple comparison test or a Kruskal–Wallis test followed by Dunn's multiple comparison test. Significance was set at $P < 0.05$. Data were plotted with GraphPad Prism 8.31 for Windows (GraphPad Software Inc, La Jolla, CA). Data from 2 to 3 independent experiments are presented as the mean ± SEM or the median and the interquartile range, unless otherwise stated.

3. Results and discussion

3.1. Analytical chemistry

The actual concentrations of carbaryl in the experimental solutions were 94–109% of the nominal values (Table 1). Moreover, the stability results reported in Table 1 show that after 24 h, the carbaryl content in the experimental solutions had decreased only 5–20%.

3.2. Environmentally relevant concentrations of carbaryl alter essential motor behaviours in zebrafish larvae

One of the most responsive endpoints prior to acute exposure to carbamates both in mammals and fish is altered motor behaviour (Gunasekara et al., 2008a). Consequently, a battery of tests including BLA, the VMR and the VSR was used to analyse the motor function of control and carbaryl-treated larvae.

A nonmonotonic concentration response (NMCR) relationship was found between carbaryl and BLA, with a significant decrease in locomotor activity in larvae exposed for 24 h to 0.06–66 ng/L carbaryl but not in those exposed to 660 ng/L carbaryl ($H(5) = 88.616$, $P = 1.4 \times 10^{-18}$) compared to that of the controls (Fig. 1A). The monoaminegic system is one of the main modulators of locomotor activity (Rico et al., 2011), and a significant decrease in BLA has been reported in zebrafish larvae treated with deprenyl, a monoamine oxidase inhibitor that increases serotonin and dopamine levels in the brains of the larvae (Faria et al., 2021b; Sallinen et al., 2009; Bellot et al., 2021). However, in a recent study by Faria et al. (2021), 7 dpf zebrafish larvae exposed for 24 h to different fenitrothion concentrations exhibited a similar decrease in BLA without any change in the monaminergic neurotransmitters, emphasizing that this system is not the only modulator of locomotor behaviour in zebrafish larvae. Interestingly, a similar NMCR relationship was found between fenitrothion and BLA in that study.

Larvae exposed to 66–660 ng/L carbaryl showed a highly significant increase in VMR ($H(5) = 47.712$, $P = 4.2 \times 10^{-8}$) (Fig. 1B). Since the VMR of zebrafish larvae integrates motor and sensory responses, several mechanisms could be involved in the effect of carbaryl on this behaviour, including damage to mechanisms involved in the detection of changes in illumination or the specific impairment of motor circuits involved in VMR. Indeed, carbaryl has been reported to induce visual system dysfunction in humans and some other animal models (El-Sherbeeny, 2001).

Table 1
Experimental concentrations of carbaryl, recoveries obtained in the SPE procedure and stability of two of the concentrations 24 h after preparation.

Nominal concentration (ng/L)	Experimental concentration (ng/L)	% Recovery	Experimental concentration (ng/L) after 24 h
0.066	$0.066 \pm 1.85 \times 10^{-4}$	99.3 ± 0.35	$0.058 \pm 5.59 \times 10^{-3}$
0.66	$0.655 \pm 2.28 \times 10^{-3}$	99.3 ± 0.35	0.577 ± 0.059
6.60	6.18 ± 0.69	93.6 ± 10.45	5.87 ± 0.15
66.00	72.06 ± 1.05	109.2 ± 1.59	58.22 ± 0.98
660.0	662.1 ± 3.37	100.3 ± 0.51	529.70 ± 7.08

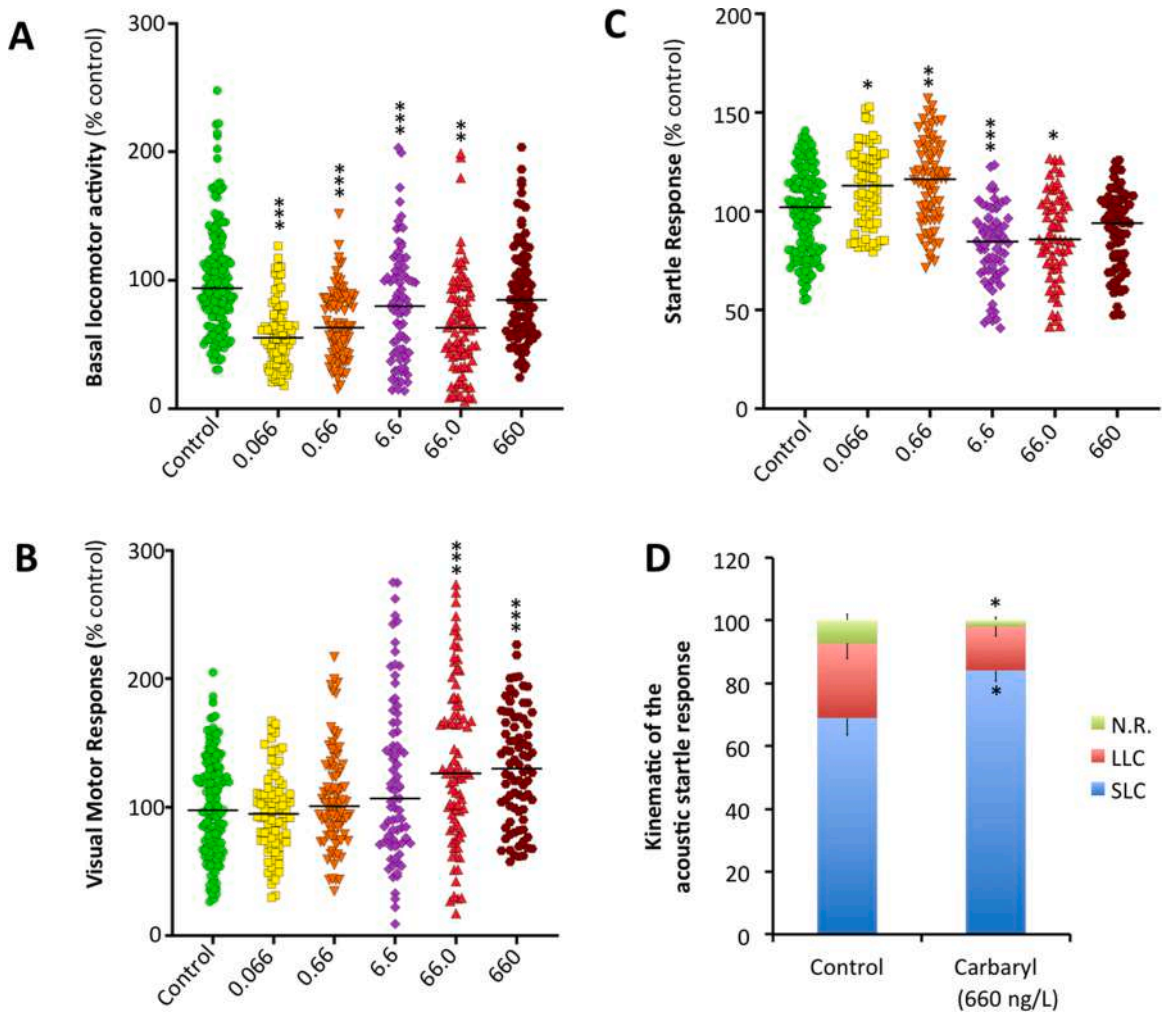


Fig. 1. Twenty-four hours exposure to a range of carbaryl concentrations commonly found in aquatic ecosystems impairs locomotor behaviour in 8 days-post fertilization zebrafish larvae. (A) Basal locomotor activity analysis shows hypoactivity of larvae exposed to 0.06–66.0 ng/L carbaryl. (B) Visual motor response is significantly increased in larvae exposed to 66–660 ng/L during the light/dark transition. (C) Analysis of the vibrational startle response, showing a significant decrease of the intensity of the startle in larvae exposed to 0.066–0.66 ng/L carbaryl, and an increase in the response in larvae exposed to 6.6–66.0 ng/L. (D) Kinematics of the acoustic/vibrational-evoked escape response show an increase in the SLC/LLC ratio in larvae exposed to 660 ng/L (LLC: long-latency C-bend; N.R.: non-responder; SLC: short-latency C-bend). Data reported as scatter plot with the median (n = 82–158 for BLA, n = 79–153 for VMR, and n = 50–51 for vibrational startle response) *p < 0.05, * *p < 0.01, * **p < 0.001; Kruskal Wallis test with Bonferroni correction; Data from 2 to 3 independent experiments.

Next, the effect of carbaryl on the VSR was analysed. Fig. 1C shows that, similar to BLA, carbaryl and the VSR have a NMCR relationship, with the magnitude of the VSR increasing in larvae exposed to 0.06–0.6 ng/L carbaryl and decreasing in individuals exposed to 6.6–66 ng/L carbaryl ($H(5) = 105.042$, $P = 1.1 \times 10^{-25}$). Interestingly, Faria et al. (2021) reported a similar relationship between fenitrothion and the VSR in 7 dpf zebrafish larvae exposed to carbaryl for 24 h.

The VMR and VSR are two ecologically relevant behaviours in the zebrafish larvae repertoire that are evoked by visual and vibrational stimuli, respectively. These behaviours integrate sensory circuits involved in stimulus detection and impulse conduction with specific circuits involved in the evoked motor response (Fero et al., 2011; Bollmann, 2019; Burgess and Granato, 2007a). Therefore, the observed effects of carbaryl on these behaviours could potentially be mediated by the effect of this chemical on sensory circuits, motor circuits or areas of the central nervous system involved in the integration of these responses. The fact that carbaryl increases motor activity in response to visual stimuli but decreases motor activity in response to vibrational stimuli strongly suggests that sensory and/or integrative circuits in the CNS, not the neuromuscular system, are the main targets of carbaryl.

Finally, the effect of carbaryl on the kinematics of the escape response evoked by an acoustic/vibrational stimulus was also analysed. In response to an acoustic/vibrational stimulus, zebrafish larvae can react with a short latency C-bend (SLC), a long latency C-bend (LLC) or provide no response to the stimulus (Burgess and Granato, 2007b). Fig. 1D shows that larvae exposed to 660 ng/L carbaryl for 24 h exhibited a significant increase in the number of individuals responding with short latency C-bends (SLCs) to the vibrational stimulus [69.75 ± 4.82% of the control larvae *vs.* 83.80 ± 4.17% of the treated larvae, n = 9 (number of assays, 9 larvae each); $P = 0.042$, Student's t test]. Moreover, there was a significant decrease in the number of larvae that did not respond to the stimulus [8.39 ± 2.31% in the control group *vs.* 1.23 ± 1.23% in the treated group, n = 9 (number of assays, 9 larvae each); $P = 0.016$, Student's t test]. Whereas the SLC response is triggered by strong vibrational stimuli detected by the otoliths and/or the lateral line, the LLC response is otolith-dependent (Fero et al., 2011; Burgess and Granato, 2007a; Privat et al., 2019). A similar effect on the kinematics of the acoustic/vibrational startle response was previously reported for fenitrothion (Faria et al., 2021). A specific effect of carbaryl exposure on the otoliths or hair cells of the otic vesicle could explain the observed decrease in LLC response found in carbaryl-exposed larvae. For instance, in *keinstein* mutants, which lack the anterior and posterior

otoliths, the LLC response, but not the SLC response, is completely abolished (Burgess and Granato, 2007a). Additional studies are needed to fully understand the effect of carbaryl exposure on vibrational startle responses.

3.3. Survival of the larvae to predator strikes is related to the vibrational startle response outcome

The VSR is an essential defensive behaviour for fish larvae, increasing their likelihood of surviving predator strikes. This response is commonly determined in laboratories by measuring the distance moved by the larvae in response to a vibrational stimulus provided by a solenoid tapping the experimental arena. However, it is difficult to predict whether the changes in the VSR of larvae measured in the above conditions are reflected in changes in survival rates after a predator attack. To assess the ecological relevance of the changes in the VSR observed in this study, we analysed the susceptibility of zebrafish larvae to predation by dragonfly nymphs, a well-established ambush predator model. The tested larvae exhibited an increased VSR (0.66 ng/L carbaryl) or a decreased VSR (66 ng/L carbaryl) compared to that of the control larvae. As Supplementary Videos S2–3 show, dragonfly nymphs attack fish larvae with extremely rapid strikes using their prehensile mask; the larvae's escape from this attack relies on the function of specific sensory-motor neuronal circuits. In the predation assay, 20%, 10% and 38% of larvae survived in the control, 0.66 ng/L carbaryl and 66 ng/L carbaryl groups, respectively. When a chi-square test of independence was performed to analyse the relationship between the VSR and survival of predator strikes, a significant relationship between the two variables was found (χ^2 (2) = 11.486, P < 0.01). Even though *Sympetrum vicinum* nymphs are allopatric predators for zebrafish larvae, the predator–prey context is still relevant (Bhattacharyya et al., 2019). The results strongly support that the altered VSR observed in carbaryl-treated larvae results in a highly relevant ecological effect, altering the survival of larvae in ambush predator attacks.

Supplementary material related to this article can be found online at doi:10.1016/j.jhazmat.2022.128563.

3.4. Predicted target profile for carbaryl

All known and predicted safety-relevant targets for carbaryl are

Table 2
Experimentally known (in ToxCast) and computationally predicted (by CLARITY; grey cells with values in italics) *in vitro* binding affinities (pK_i) between carbaryl and protein targets associated with potential behaviour-altering side effects. Experimental *in vitro* data from predictions are given in bold and in parenthesis.

Protein	Gene name	pK_i	Side effect
Acetylcholinesterase (green rice leafhopper)	AChE	7.5	Seizures
Carboxylic ester hydrolase (house fly)	CEH	6.7	-
Acetylcholinesterase (homo sapiens)	ACHE	5.7	Seizures
Cytochrome P450 3A4	CYP3A4	5.5	-
Aryl hydrocarbon receptor	AHR	5.3	-
GLI zinc finger 3	GLI3	5.0	-
Vitamin D receptor	VDR	4.9	Affects calcium levels
Progesterone receptor	PGR	4.6	-
Androgen receptor	AR	4.5	Increased hostility
Estrogen receptor 2	ESR2	active	-
Kallikrein-related peptidase 5	KLK5	active	-
5-hydroxytryptamine receptor 2B	HTR2B	*7.1* (**5.4**)	Cardiac effects
Beta-1 adrenoceptor	ADRB1	*6.4*	Affects heart rate
Sodium channel protein type 2 subunit alpha	SCN2A	*6.2*	Seizures
Glutamate receptor ionotropic, kainate	GRIK	*6.2*	Seizures
Alpha-2B adrenoceptor	ADRA2B	*6.0* (**52%** at 10μM)	Vasoconstriction
Adenosine receptor A1	ADORA1	*active*	Bradycardia
Glutamate receptor ionotropic, AMPA	GRIA	*active*	Seizures

listed in Table 2. Arguably, the main toxicological mode of action for carbaryl is its known interaction with AChE. The binding affinity for this canonical target is, however, strongly dependent on the species. While the affinity of carbaryl for human AChE is in the low micromolar range (pKi = 5.7), its affinity for the green rice leafhopper AChE orthologue is almost two orders of magnitude more potent (pKi = 7.5). Additionally, carbaryl is known to be weakly active on several nuclear receptors, with binding affinities above 10 μM. Thus, there seems to be a reasonable correlation between human and zebrafish EC_{20} activity values (Neale et al., 2020).

The seven predicted human targets of carbaryl associated with potential cardiotoxic and neurotoxic side effects that could alter behaviour upon exposure is also provided in Table 2. Only safety-relevant targets with prediction affinities below 1 μM ($pK_i \geq 6$) and qualitative "active" predictions were considered at this stage. Of these, affinity for the serotonin 2B receptor (HTR2B), the alpha-2B and beta-1 adrenoceptors (ADRA2B and ADRB1), and adenosine A1 receptor (ADORA1) are linked to various cardiotoxicity endpoints (Mamoshina et al., 2021; Funakoshi et al., 2006; Huang et al., 2011), whereas affinity for the sodium channel protein type 2 subunit alpha (SCN2A) and glutamate (kainite) ionotropic receptors and AMPA (GRIK and GRIA) may likely lead to seizures (Reynolds et al., 2020; Bowie, 2022; Negrete-Díaz et al., 2021).

3.5. *In silico predictions were validated by in vitro assays*

The most relevant carbaryl interactions predicted by the *in silico* profiling analysis were validated by *in vitro* competition binding assays (Table 2). As Supplementary Table ST3 shows, carbaryl did not significantly inhibit the specific binding of [^{3}H]-AMPA (GRIA1), [^{3}H]-kainic acid (GRIK), [^{3}H]-DPCPX (ADORA1), [^{3}H]-oestradiol (ESR2) or [^{3}H] methyltrienolone (AR). Moreover, as Supplementary Fig. S2 shows, carbaryl did not activate the beta-1 adrenergic receptor (ADRB1). However, the low micromolar affinity ($pK_i = 5.4$) between carbaryl and HTR2B was experimentally confirmed, with carbaryl behaving as an HTR2B antagonist in this assay. Additionally, concentrations of 10 μM carbaryl caused 52% and 58% inhibition of the specific binding of [^{3}H]-rauwolscine and acetylcholine to ADRA2B and AChE, respectively.

Finally, no interaction between carbaryl and the voltage-gated sodium channel SCN2A was found by using a QPatch electrophysiological platform (Supplementary Table ST4).

3.6. *Validating in silico predictions with in vivo assays in zebrafish larvae*

Once the interactions of AChE, HTR2B and ADRA2B with carbaryl were validated in the *in vitro* assays, we determined the potential role of these molecular targets in the observed adverse effects of carbaryl in zebrafish larvae.

3.6.1. *AChE*

Inhibition of AChE activity is the most accepted molecular initiating event (MIE) of carbaryl neurotoxicity. Therefore, AChE activity was determined in larvae exposed to a wide range of carbaryl concentrations (from 0.66 ng/L to 660 μg/L) to confirm whether the observed behavioural changes in larvae exposed to environmentally relevant concentrations of carbaryl were related to the inhibition of this enzyme. As Fig. 2 shows, carbaryl concentrations in the range of 0.66 ng/L to 66 μg/L failed to significantly inhibit AChE activity ($F_{4,38} = 1.164$, $P = 0.342$). These results demonstrate that the neurobehavioural effects of carbaryl on zebrafish larval behaviour are due to AChE-independent mechanisms. Interestingly, the neurobehavioural changes in zebrafish larvae exposed to environmental concentrations of fenitrothion were also reported to be due to AChE-independent mechanisms (Faria et al., 2021).

3.6.2. *Androgen receptors*

The US EPA's ToxCast™ database identifies the androgen receptor (AR) as a target for carbaryl (https://comptox.epa.gov/dashboard).

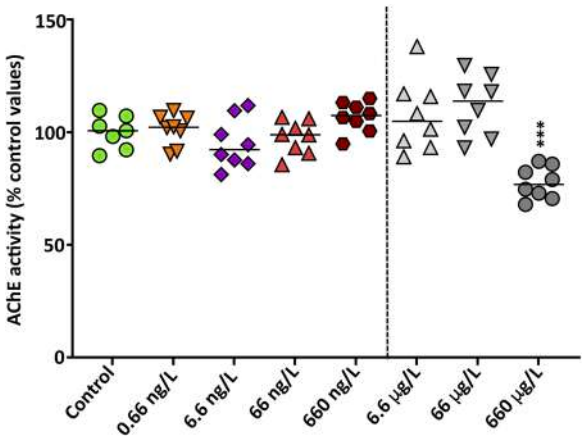
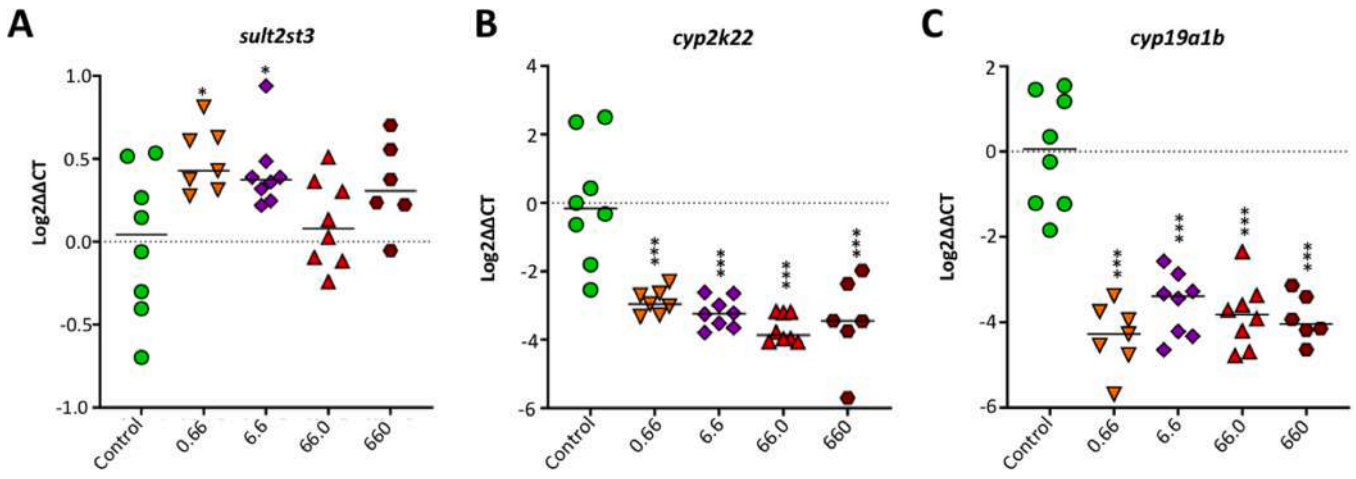


Fig. 2. Acetylcholinesterase (AChE) activity in 8 days post-fertilization zebrafish larvae control and exposed to different concentrations of carbaryl. Data reported as scatter plot with the median (n = 8–10; P > 0.05; one-way ANOVA with Dunnett's multiple comparison test; Data from 2 independent experiments).

Moreover, in a recent study, the exposure of zebrafish larvae to environmentally relevant concentrations of fenitrothion (another chemical targeting AR) not only resulted in the upregulation of the androgen-regulated genes *sult2st3* and *cyp19a1b* but also in behavioural changes strikingly similar to those we observed for carbaryl. Therefore, even though the interaction of carbaryl with human AR was not confirmed by the *in vitro* validation, we explored the potential involvement of the androgenic pathway in the observed behavioural changes in zebrafish. First, the effect of carbaryl on the expression of three classical transcriptional markers of androgenicity (*sult2st3*, *cyp2k22* and *cyp19a1b*) was analysed. Fig. 3A shows that carbaryl had a nonmonotonic effect on *sult2st3* expression ($F_{4,32} = 30.621$, $P = 1.57 \times 10^{-10}$), inducing a significant increase in transcript levels after exposure to the lowest concentrations of carbaryl (0.66 and 6.6 ng/L) and then returning to the control levels at higher concentrations. In contrast, carbaryl exposure significantly decreased transcript levels of *cyp2k22* ($F_{4,32} = 3.784$, $P = 0.012$) and *cyp19a1b* ($F_{4,32} = 16.705$, $P = 1.73 \times 10^{-7}$) throughout the whole range of carbaryl concentrations used in this study (Fig. 3B-C). These results contrast with the recently reported findings that *cyp2k22* and *cyp19a1b* were upregulated in zebrafish larvae exposed to low concentrations of fenitrothion (Faria et al., 2021). The fact that carbaryl downregulates the expression in two out of three androgen-responsive genes strongly suggests that carbaryl is not acting as an agonist of AR but perhaps as an antagonist of this nuclear receptor.

To clarify the relationship between activation of androgenic and antiandrogenic pathways and subsequent behavioural effects, 7 dpf zebrafish larvae were exposed for 24 h to a prototypic agonist (500 nM testosterone) and antagonist (20 μM nilutamide) of AR, and their effects on *sult2st3*, *cyp2k22* and *cyp19a1b* expression and behaviour were analysed and compared with those of the effects of carbaryl. The selected concentrations of each chemical were based on previous reports (Fetter et al., 2015; Jarque et al., 2019). Consistent with these reports, 24 h of exposure to these AR modulators significantly altered the expression of *sult2st3* ($F_{2,21} = 105.071$, $P = 1.15 \times 10^{-11}$), *cyp2k22* ($F_{2,21} = 142.557$, $P = 6.05 \times 10^{-13}$) and *cyp19a1b* ($F_{2,21} = 317.539$, $P = 2.02 \times 10^{-16}$). Whereas exposure to 500 nM testosterone resulted in a significant increase in the expression of the three transcriptional markers (Supplementary Fig. S3), exposure to 20 μM nilutamide, a concentration reported to counteract the effects of testosterone on androgen-responsive genes, had no effect on *sult2st3* and *cyp2k22* expression. Interestingly, exposure to nilutamide increased the expression of *cyp19a1b* (an oestrogen- and androgen-regulated gene), although at a lower level than testosterone. Moreover, the effects of 500 nM



D

	sult2st3	cyp2k22	cyp19a1b
0.66 ng/L Carbaryl	↑	↓	↓
6.6 ng/L Carbaryl	↑	↓	↓
66 ng/L Carbaryl	NE	↓	↓
660 ng/L Carbaryl	NE	↓	↓
500 nM Testosterone	↑	↑	↑
20 uM Nilutamide	NE	NE	↑

E

	BLA	VMR	VSRA
0.066 ng/L Carbaryl	↓	NE	↑
0.66 ng/L Carbaryl	↓	NE	↑
6.6 ng/L Carbaryl	↓	NE	↓
66 ng/L Carbaryl	↓	↑	↓
660 ng/L Carbaryl	↓	↑	NE
500 nM Testosterone	NE	NE	NE
20 uM Nilutamide	NE	↑	↑

Fig. 3. Assessment of the potential involvement of the androgenic or anti-androgenic pathways on the carbaryl effects. Effect of carbaryl on the expression of *sult2st3* (A), *cyp2k22* (B) and *cyp19a1b* (C) in 8 dpf zebrafish larvae. (D) Changes in the expression of three selected androgen-regulated genes in 8 dpf zebrafish larvae after 24 h exposure to carbaryl, the androgenic agonist testosterone and the antiandrogenic nilutamide. (E) Changes in the basal locomotor activity (BLA), visual motor response (VMR) and vibrational startle response (VSRA) in 8 dpf zebrafish larvae after 24 h exposure to carbaryl, the androgenic agonist testosterone and the antiandrogenic nilutamide Data reported as scatter plot with the median (n = 6–8; P > 0.05; one-way ANOVA with Dunnett's multiple comparison test; Data from 2 independent experiments).

testosterone (an AR agonist) and 20 μM nilutamide (an AR antagonist) on BLA, the VMR and VSR of zebrafish larvae were examined and compared with the effects found for carbaryl (Supplementary Fig. S4). Whereas exposure to testosterone had no effect on any of the analysed behaviours, nilutamide induced a significant increase in the VMR ($F_{2123} = 7.073$, $P = 0.001$) and VSR ($H(2) = 20.810$, $P = 3.03 \times 10^{-5}$). As Fig. 3D and E shows, the carbaryl-induced changes in gene expression and behaviour are not consistent with the effects of prototypic androgenic and antiandrogenic compounds, suggesting that the effect of carbaryl on zebrafish behaviour is AR-independent. Moreover, the information obtained on the effect of androgenic pathway modulation on zebrafish larvae behaviour only allows us to discard this potential MIE for carbaryl neurotoxicity but also allows us to re-evaluate the involvement of this pathway in the neurotoxicity induced by other pollutants. For instance, after finding that *sult2st3* and *cyp19a1b* were upregulated, Faria et al. Faria et al. (2021) recently proposed that the potential MIE of fenitrothion that led to neurobehavioural effects in zebrafish larvae was an agonistic effect on ARs. Although larvae exposed to testosterone in our study exhibited similar changes in the expression of *sult2st3* and *cyp19a1b*, this prototypic agonist of AR failed to reproduce any of the neurobehavioural effects induced by fenitrothion. This finding demonstrates that even if fenitrothion has an agonistic effect on zebrafish ARs, androgenic activation is not directly involved in the fenitrothion-induced neurobehavioural effects in zebrafish larvae.

3.6.3. Alpha-2B adrenoceptor (ADRA2B) and serotonin 2B receptor (HTR2B)

CLARITY v5 predicted that ADRA2B was a potential molecular target of carbaryl, and further *in vitro* competition binding assays confirmed the interaction of ADRA2B with this insecticide. α2B-adrenoceptors are well-known regulators of cardiovascular function in mammals. Therapeutic doses of α2B-adrenergic agonists reduce heart rate (HR), but α2B-adrenergic antagonists increase HR (Szabadi and Bradshaw, 1996; Arnar et al., 2007). ADRA2B has been related to spatial working memory and the analgesic pathway for nitrous oxide (Philipp et al., 2002). In adult zebrafish, *adra2b* expression has been reported in both the brain and heart (Ruuskanen et al., 2005). Therefore, to validate interaction of zebrafish α2B-adrenoceptors with carbaryl *in vivo*, the effect of this chemical on larval HR was analysed in a range of concentrations from 60 pg/L to 66 ng/L carbaryl (0.3–328.0 pM). As Fig. 4A shows, carbaryl exposure resulted in a significant increase in larval HR (positive chronotropy; $F_{4,79} = 159.381$, $P = 5.4 \times 10^{-37}$). The lowest carbaryl concentration used, 60 pg/L (0.3 pM), increased larval HR by approximately 16% over that of the corresponding controls, and the effect reached a steady state at 600 pg/L, with an increase of approximately 38% over that of the corresponding controls. Thus, the effect of carbaryl on HR in zebrafish larvae is consistent with the expected effect of an ADRA2B antagonist in mammals. However, to confirm that picomolar concentrations of carbaryl induced a positive chronotropic effect on larval zebrafish HR by blocking the α2B-adrenoceptor, the effect of yohimbine, a widely used α2-adrenergic antagonist, on zebrafish HR was tested. We exposed 7 dpf zebrafish larvae to 0.1 μM, 1.0 μM and 10 μM yohimbine for 24 h. Consistent with the reported effect in mammalian models, yohimbine exposure induced a significant increase in HR in zebrafish larvae ($F_{3,68} = 45.621$, $P = 2.84 \times 10^{-16}$; Fig. 4B). The HR after yohimbine exposure was 28–33% greater than the control values. These results demonstrate that the effect of picomolar concentrations of carbaryl on zebrafish cardiac function is consistent with the effect of a prototypical ADRA2B antagonist on this endpoint. Then, the next step was to determine if ADRA2B antagonism was the MIE of the observed effects on behaviour in zebrafish larvae. To this end, 7 dpf larvae were exposed to 100 nM yohimbine for 24 h, and BLA, the VSR and the VMR were analysed. As Fig. 5 shows, larvae exposed to the ADRA2B antagonist exhibited altered behaviour characterized by significantly reduced BLA ($U(N_{control}=95, N_{yohimbine}=144)= 5762.50$, $z = -2.06$, $P = 0.039$) and VSR ($t(234) = 2.738$, $P = 0.0067$). However, no differences in the VMR were found between control and exposed larvae ($t(237) = -0.036$, $P = 0.971$). Li et al. Li et al. (2015) also assessed the effect of yohimbine on larval zebrafish behaviour. Although these authors also found a significant decrease in BLA and altered VMR, they used higher yohimbine concentrations (28–565 μM) and shorter exposures (behaviour determined 10 min after exposure), making a direct comparison with our result impossible. Interestingly, the behavioural profile observed in the zebrafish larvae exposed to the ADRA2B antagonist yohimbine was a behavioural phenocopy of that observed in larvae exposed to 33 pM (6.6 ng/L) carbaryl (Fig. 1). While the results presented above strongly suggest that the antagonistic effect of carbaryl on ADRA2B is the MIE for the observed behavioural changes, it is important to consider that although yohimbine is commonly used as a "selective" α2B-adrenergic antagonist, it is also an antagonist of different serotonin receptors, including HTR2B (Ruuskanen et al., 2005).

CLARITY v5 also predicted that the serotonin 2B receptor was a molecular target of carbaryl, and during further *in vitro* validation, an antagonistic interaction of carbaryl with this serotonin receptor was confirmed. Stimulation of HTR2B has been linked to chronic valvular heart disease (Arnoux and Ayme-Dietrich, 2021), and inactivation of HTR2B receptors during development causes cardiac defects (Nebigil et al., 2001), but no data are currently available on the effect of HTR2B modulators on fish heart rate. To determine whether HTR2B antagonism also contributes to the potent increase in HR observed in carbaryl-exposed larvae, 7 dpf zebrafish larvae were exposed for 24 h to 0.1 μM and 1.0 μM SB204741, a specific HTR2B antagonist, and the resulting HR was analysed. As Fig. 4C shows, exposed larvae exhibited a mild to moderate increase in HR ($F_{2,75} = 11.940$, $P = 0.000031$). The largest effect on HR occurred at 1.0 μM SB204741, with an increase of 15% over that of the corresponding controls. While the effect of blocking HTR2B on HR was only moderate, its effect on behaviour could be higher. Therefore, 7 dpf zebrafish larvae were exposed for 24 h to 100 nM SB204741, a concentration with only a very mild effect on HR, and the effects on BLA, the VSR and the VMR were analysed. As Fig. 5 shows, larvae exposed to the HTR2B antagonist exhibited a potent (Mdn = 55.91%) reduction in BLA ($U(N_{control}= 70, N_{yohimbine}= 95)= 1839.500$, $z = -4.898$, $P = 9.70 \times 10^{-7}$). The HTR2B antagonist moderately decreased VSR ($U(N_{control}= 76, N_{yohimbine}= 124)= 3414.000$, $z = -3.267$, $P = 0.001$). Finally, no significant effect on VMR was found in the SB204741-exposed larvae ($t(238) = 1.040$, $P = 0.299$).

To determine whether the observed effects on these receptors were also reflected at the transcriptional level, the expression of *adra2b* and *htr2b* was analysed in zebrafish larvae exposed to 0.66–660 ng/L

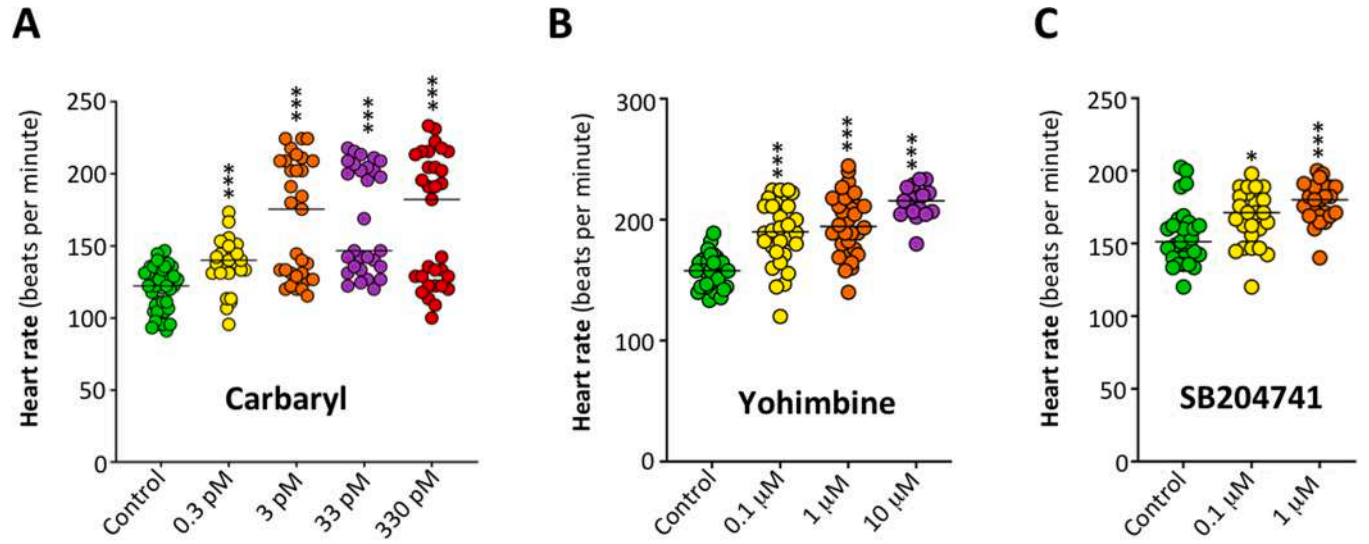


Fig. 4. Effect of 24 h exposure to carbaryl (0.3–330 pM), yohimbine (0.1–10 μM) and SB204741 (0.1–1.0 μM) on cardiac activity in zebrafish larvae. Data reported as scatter plot with the median of the beats per minute (n = 29–43 for carbaryl, 17–21 for yohimbine and 25–29 for SB-204741; one-way ANOVA with Dunnett's multiple comparison test; *p < 0.05, **p < 0.01, ***p < 0.001; Data from 2 independent experiments).

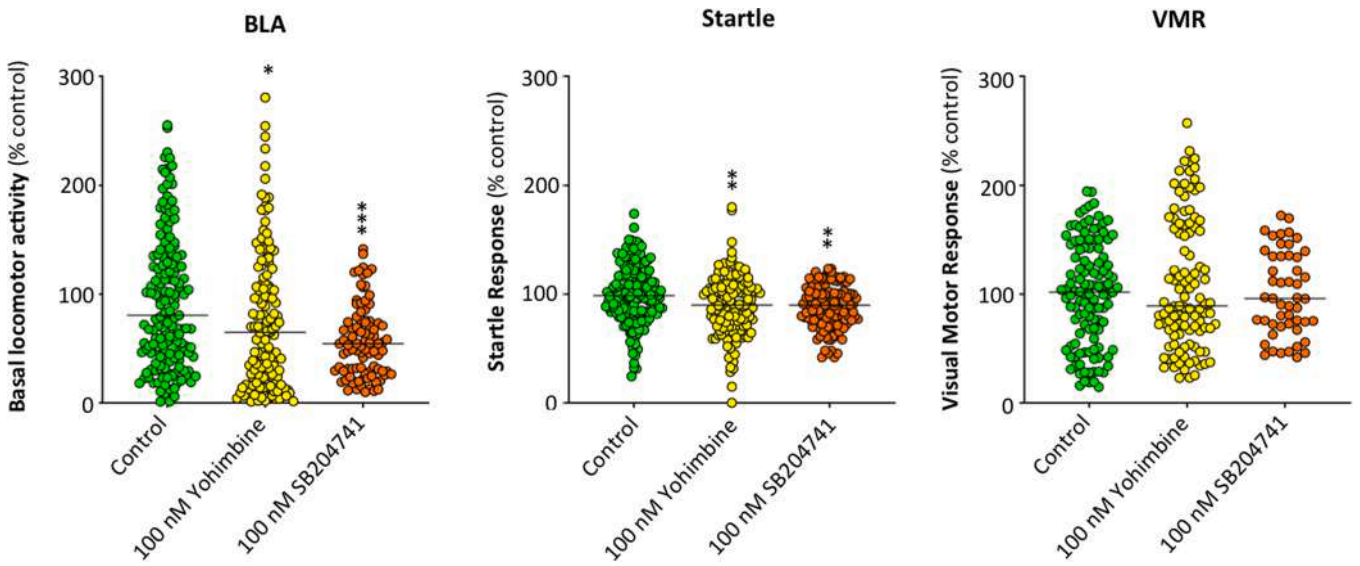


Fig. 5. Behavioural effects of 24 h exposure to 0.1 μM yohimbine and 0.1 μM SB204741 in 8 dpf zebrafish larvae. Data reported as scatter plot with the median (n = 95–165 for BLA, n = 124–169 for Startle, and n = 50–117 for VMR) *p < 0.05, * *p < 0.01, * **p < 0.001; Student's *t*-test or Mann-Whitney test; Data from 2 to 3 independent experiments.

carbaryl from 7 to 8 dpf. No significant effects were observed on the expression of *adra2b* ($F_{4,25} = 0.442$, $P = 0.777$) and *htr2b* ($F_{4,25} = 0.963$, $P = 0.445$) in the exposed larvae (Supplementary Fig. S5).

Fig. 6 summarizes the effect of the 24-h exposure to carbaryl, yohimbine and SB204741 on HR, BLA, startle and the VMR. The most consistent effects of carbaryl at varying concentrations were the positive chronotropic effect on HR and the significant decrease in BLA. Moreover, in the 33–330 pM (0.6–66 ng/L) range of concentrations, carbaryl induced a significant decrease in the intensity of the startle evoked by a vibrational stimulus. When both ADRA2B and HTR2B were blocked by yohimbine, a phenotype strikingly similar to that induced by 33 pM carbaryl was obtained. However, the fact that when only HTR2B was blocked (with SB204741), the main effect was on BLA strongly suggests that the effect of carbaryl on zebrafish larvae involves the interaction of both monoaminergic receptors, with the ADRA2B antagonism playing a more relevant role on HR and HTR2B antagonism in decreasing BLA.

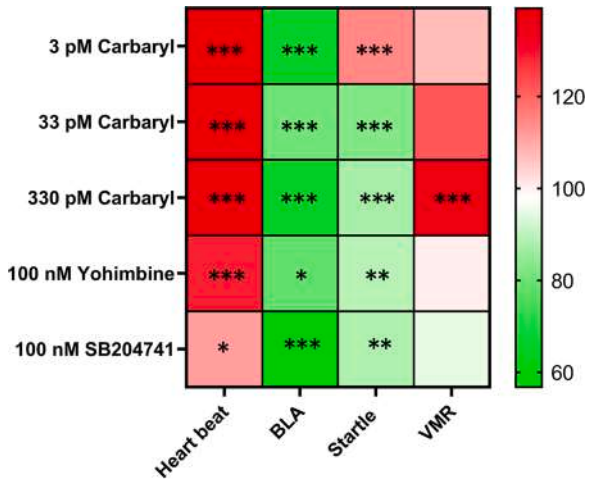


Fig. 6. Heat map diagram showing the changes in heart rate, basal locomotor activity (BLA), vibrational startle response (Startle) and visual motor response (VMR) observed in 8 days post-fertilization zebrafish larvae after 24 h treatment with carbaryl (3–330 pM), 0.1 μM yohimbine and 0.1 μM SB204741. Colours in the heat map represent the deviation from the control larvae.

3.7. Additional monoaminergic effects of carbaryl

As ADRA2B and HTR2B are two receptors of the monoaminergic system, which has an essential role in modulating behaviours, additional changes in this neurotransmitter system were explored by analysing and gene expression and the neurochemical profile in the exposed larvae.

As Fig. 7 shows, while the levels of dopamine (DA) in the exposed larvae remained unchanged, carbaryl significantly altered the levels of tyrosine ($H(4) = 36.491$, $P = 2.3 \times 10^{-7}$) and L-DOPA ($F_{4,47} = 8.511$, $P = 3.0 \times 10^{-5}$). However, tyrosine levels increased in larvae exposed to 0.066–6.6 ng/L carbaryl and L-DOPA levels increased in larvae exposed to 0.66–66 ng/L carbaryl. The levels of norepinephrine (NE), a physiological ligand for ADRA2B, also remained unchanged after carbaryl exposure ($F_{4,47} = 1.088$, $P = 0.373$). Additional information on DA metabolites is provided in the Supplementary Results and Supplementary Fig. S6.

When the serotonergic system was analysed, there was a significant effect of carbaryl exposure on tryptophan ($H(4) = 29.173$, $P = 7.2 \times 10^{-6}$), serotonin ($F_{4,47} = 13.655$, $P = 4.4 \times 10^{-4}$) and 5-HIAA ($F_{4,47} = 13.340$, $P = 2.4 \times 10^{-7}$) (Fig. 7). While the levels of tryptophan increased, the levels of serotonin and its degradation product 5-HIAA decreased. To better understand the effect of carbaryl on the serotonergic system, the expression of the main genes involved in the synthesis (*tph1a*, *tph1b*), transport (*slc18a2*, which encodes VMAT2, and *slc6a4*, which encodes SERT) and degradation (*mao*) of serotonin was analysed. As Fig. 7 shows, the only effect found in the expression of these genes after carbaryl exposure was for *slc6a4* ($F_{4,31} = 3.982$, $P = 0.010$) and *mao* ($F_{4,30} = 13.167$, $P = 2.63 \times 10^{-6}$) expression. Interestingly, the US EPA's ToxCast™ database (https://comptox.epa.gov/dashboard) identifies the serotonin transporter (SERT, the product of *slc6a4*) and monoamine oxidase (MAO, the product of *mao*) as targets of carbaryl. An inverted U-shaped nonmonotonic concentration–response curve described the association between *slc6a4* expression and carbaryl levels, with an important downregulation of *slc6a4* expression at the lowest carbaryl concentration and no effect at higher concentrations (Fig. 7). In contrast, carbaryl exposure resulted in a significant decrease in the expression of *mao* at all tested concentrations. Therefore, the potential role of MAO was further explored by analysing MAO activity. As Fig. 7B shows, 5 μM (1.1 mg/L) deprenyl (positive control) induced a total abolition of MAO activity, yet none of the carbaryl concentrations tested had any effect on this activity ($F_{4,20} = 1.191$, $P = 0.345$). These results

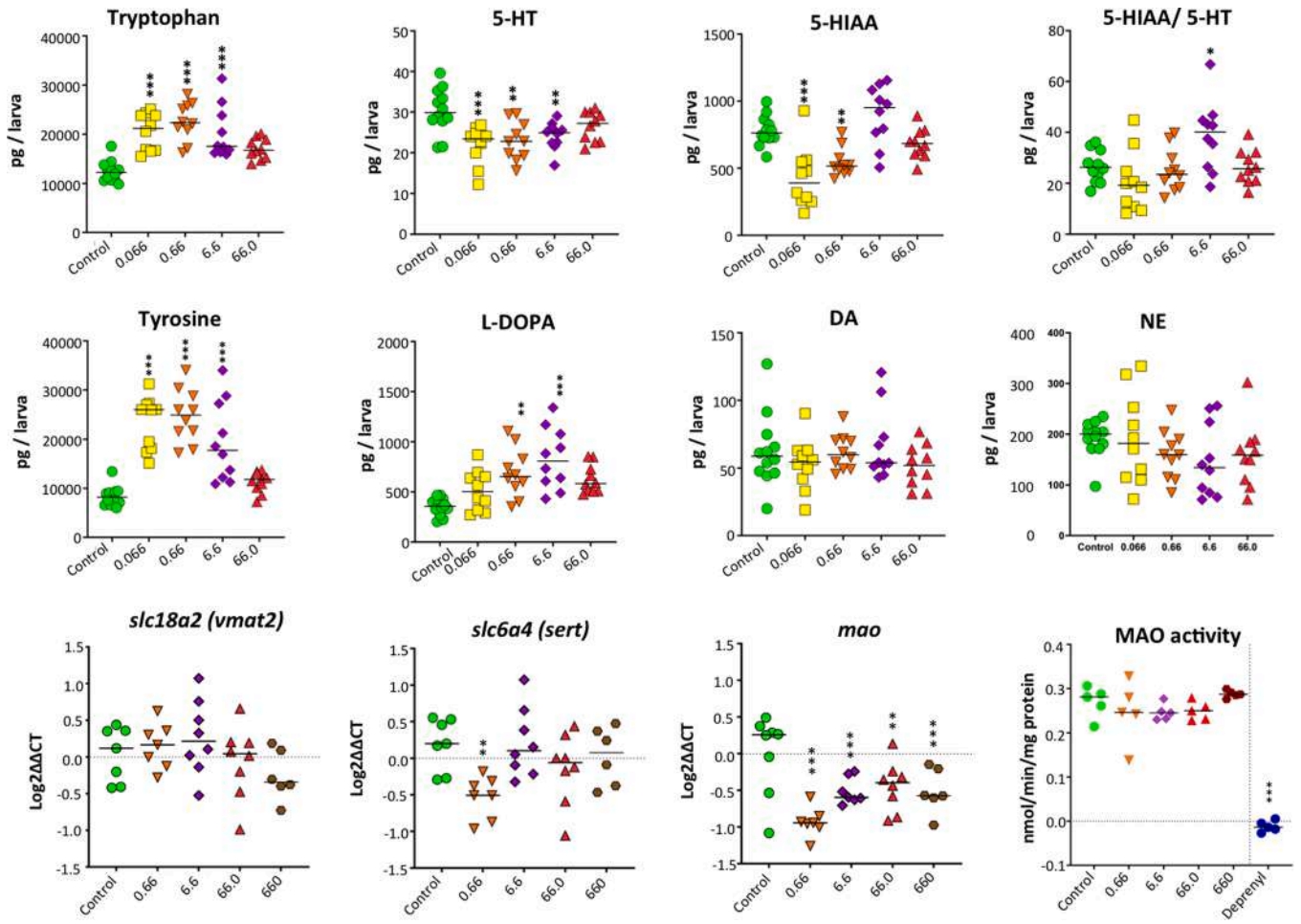


Fig. 7. Effects of 24 h exposure to environmental concentrations of carbaryl on the monoaminergic system of zebrafish larvae. Data reported as scatter plot with the median, * $P < 0.05$, * * $P < 0.01$, * ** $P < 0.001$; One way ANOVA followed by Dunnett's multiple comparison test was used for L-DOPA, DA, NE, 5-HT, 5-HIAA/5-HT, *slc18a2*, *slc6a4*, *mao* and MAO activity, whereas Kruskal Wallis test with Bonferroni correction was used for tyrosine, tryptophan, and 5-HIAA; n = 10–12 for neurochemical analyses, n = 6–8 for gene expression analyses, n = 5 for MAO activity analyses. Data from 2 independent experiments.

demonstrate that the observed effects of carbaryl on zebrafish larval behaviour were also MAO-independent.

The observed changes in the neurochemical profile of the serotonergic system might be explained as an inhibitory effect of carbaryl on tryptophan hydroxylase (TPH), the key and rate-limiting enzyme in the synthesis of 5-HT. Although no significant effect on *tph1a* ($F_{4,25} = 0.889$, $P = 0.485$) and *tph1b* ($F_{4,25} = 0.579$, $P = 0.680$) expression was found in carbaryl-exposed larvae (Supplementary Fig. S7), we could not reject a direct inhibitory effect of carbaryl on TPH activity.

In contrast to the results presented in the current manuscript, other reports have shown a relationship between carbaryl exposure and increased levels of NE, 5-HT and 5-HIAA in the mammalian brain (Hassan and Santolucito, 1971; Kumar Ray and Kanti Poddar, 1990). However, all these studies used high carbaryl doses that also inhibited AChE instead of the picomolar concentrations used in the present study. Moreover, in recent studies characterizing the neurobehavioural phenotype of zebrafish larvae treated with modulators of the serotonergic system (Faria et al., 2021a; Sallinen et al., 2009), larvae exposed to the TPH inhibitor p-chlorophenylalanine (PCPA) exhibited increased BLA and startle, whereas those exposed to deprenyl, an MAO inhibitor, exhibited a decrease in BLA, the VMR and startle. However, it is important to note that deprenyl-treated larvae presented increased serotonin levels, while those treated with PCPA exhibited serotonin levels similar to the control (Faria et al., 2021a; Sallinen et al., 2009). Although it is not possible to directly compare the observed decrease in serotonin levels in larvae exposed to carbaryl with those exposed to PCPA, these results show that the behavioural phenotype exhibited by carbaryl-treated larvae is not fully explained by the observed changes in total serotonin levels.

3.8. Environmental risk assessment consequences

U.S. EPA ambient water quality criteria for carbaryl have been fixed at 2.1 μg/L for freshwater aquatic organisms, considering that under this level, carbaryl is safe (U. States, 2012). However, in the present study, we found a consistent impairment of ecologically relevant behaviours of fish larvae, such as BLA, the vibrational-evoked escape response, and the VMR, at carbaryl concentrations 35,000, 3182 and 32 times lower, respectively, than the EPA freshwater quality criteria for this insecticide. In fact, concentrations impairing BLA and the startle response are in the low range of environmental levels commonly found in indirectly exposed aquatic ecosystems (Wilson and Foos, 2006; Fytianos et al., 2006; Terzopoulou et al., 2015; Herrero-Hernández et al., 2017; Allinson et al., 2016). Altered behaviour in larvae has detrimental fitness consequences. For instance, in the range of concentrations from 0.066 ng/L to 66 ng/L carbaryl, the increased response of larvae to environmental stimuli (light, vibrations) coupled with their increased heart rate will consume more energy, which will thus be unavailable for other physiological processes. Furthermore, we have demonstrated a significant relationship between the acoustic/vibrational startle response and larval survival to predator strikes (Fero et al., 2011). Therefore, the changes in this behaviour resulting from exposure to

neurotoxic pollutants may have dramatic effects on larval survival, leading to fish population decline and severe impacts on ecosystems (Weis et al., 2001). According to the data presented in this study and following the Environmental Risk Assessment guidelines (Report of the OECD Workshop on Environmental Hazard/risk Assessment, 2002), the environmental safety concentrations of carbaryl for fish should be lower than 0.06 ng/L.

Our study indicated that AChE inhibition is not the only relevant MoA for carbaryl, which has implications for environmental risk assessment. Moreover, it is worth noting that some effects of carbaryl, such as moderate behavioural changes, are sublethal in the laboratory but could lead to lethality in the wild. We found that at environmentally relevant concentrations, carbaryl disrupts the monoaminergic system (the adrenergic and serotonergic signalling pathways), which regulates complex behaviour and cardiac activity in fish larvae.

CRediT authorship contribution statement

Melissa Faria: Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Writing – original draft, Writing – review & editing. **Marina Bellot:** Data curation, Investigation, Writing – review & editing, **Juliette Bedrossiantz:** Data curation, Methodology, Investigation, Visualization, Writing - review & editing. **Jonathan Ricardo Rosas Ramírez:** Data curation, Investigation. **Eva Prats:** Data curation, Formal analysis, Investigation, Writing – review & editing. **Natalia Garcia-Reyero:** Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Cristian Gomez-Canela:** Data curation, Formal analysis, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Jordi Mestres:** Methodology, Software, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Xavier Rovira:** Methodology, Software, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Carlos Barata:** Writing – original draft, Writing – review & editing. **Leobardo Manuel Gómez Oliván:** Writing – review & edit. **Amadeu Llebaria:** Conceptualization, Writing – original draft, Writing – review & editing. **Demetrio Raldua:** Conceptualization, Data curation, Formal analysis, Investigation, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.128563.

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