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Development of a vibrational startle response assay for screening environmental pollutants and drugs impairing predator avoidance

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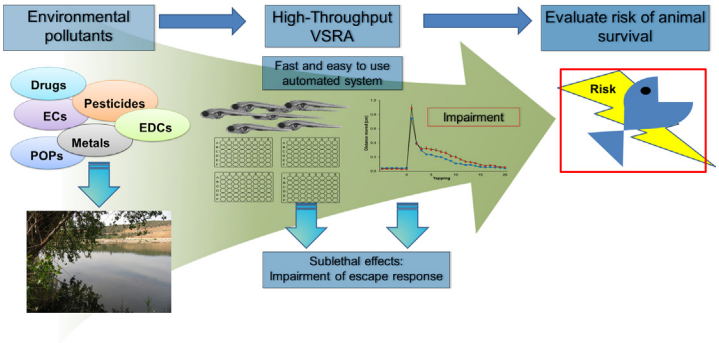
HIGHLIGHTS

- The new method enables in vivo medium- to high-throughput screening of pollutants.
- Hallmark criteria for escape response habituation were met.
- Pharmacological modulation of escape response habituation similar to mammal species
- The new method proves sensitive to different concentrations of environment pollutants.

GRAPHICAL ABSTRACT



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ABSTRACT

The present paper describes the vibrational startle response assay (VSRA), a new robust, simple and automated in vivo medium- to high-throughput procedure for assessment of the escape response and its habituation in zebrafish larvae. Such behaviors enable fish larvae to escape from predator strikes in aquatic ecosystems. The assay is based on measuring the distance moved by each larva during the startle response evoked by repetitive vibrational stimuli. The iterative reduction observed in the response to a series of tapping stimulus in VSRA met the main criteria of habituation. Subsequently, the analysis of concordance using a battery of neuroactive compounds modulating different neurotransmitter systems demonstrated that the results of VSRA are highly predictive of the effects on other vertebrates. Finally, as a proof of concept, VSRA was used to test two relevant environmental pollutants at different concentrations. The results demonstrated that VSRA is suitable for concentration-response analysis of environmental pollutants, opening the possibility to determine the potency and the associated hazard of impaired escape response for the different compounds. Therefore, we suggest that VSRA could be a valuable tool for screening of chemical compounds capable of compromising predator avoidance behavior.

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1. Introduction

In natural conditions predation is one of the main causes of mortality in feral fish, especially during the larval stage (Houde and Hoyt, 1987). As a part of an innate behavioral repertoire enabling larvae to escape from predator strikes, they respond to abrupt acoustic/vibrational stimuli with a fast C-bend followed by a bout of high-amplitude and low frequency fast swimming (Fero et al., 2011). Two modes of C-bend response have been identified according to latency. Whereas short latency C-bend (SLC) occurs within 15 ms of the stimulus, long latency C-bend (LLC) is initiated 20–60 ms after the stimulus (Fero et al., 2011). SLC response is regulated by a sensory motor axis that integrates auditory and vibrational information and transduces these stimuli into musculoskeletal activation via a bilateral pair of giant reticulospinal neurons in the hindbrain, the Mauthner cells (Painter et al., 2009). Because of their short latency and explosive speed of the movement, SLC responses are similar to the startle responses in higher vertebrates (Fero et al., 2011).

Habituation is a primitive form of implicit learning. The animal first responds to a new stimulus and, if the stimulus is neither beneficial nor harmful, animal learns, after repeated exposure, to ignore it (Kandel, 1991). Habituation of the escape response results essential for aquatic organisms, as repeated unnecessary escape responses reduce foraging and result in an increase in the predation risk by at least two different ways (Fields and Yen, 1997). On one hand, escape response supposes a high energetic cost, and repeated escape responses will result in exhausted organisms, making them more susceptible to predation. Moreover, unnecessary escape responses attract the attention of both visual and mechanoreceptive predators (Batabyal et al., 2017; Fields and Yen, 1997; Killen and Brown, 2006). Short-term habituation of C-startle response occurs when larvae is exposed to repeated stimulation at short interstimulus intervals (ISIs), with the corresponding Mauthner cell responding only to the few first stimuli, and failing then to elicit a Mauthner spike (Park et al., 2018). As a result, SLC responsiveness to the acoustic/vibrational stimuli diminishes extremely rapidly during short-term habituation.

Currently, the available information about potential adverse effects of environmental pollutants present in aquatic ecosystems on the C-startle response and habituation in fish larvae is very scarce. To our knowledge, only eight environmental pollutants have been tested to determine the effects on the C-startle in fish, and the results indicated that fish exposed to seven of these chemicals were more susceptible to predation (Carlson et al., 1998). Moreover, although it has been demonstrated that exposure to some drugs alters habituation of C-startle evoked by acoustic stimulus in fish larvae (Best et al., 2008; Marsden and Granato, 2015; Roberts et al., 2016; Wolman et al., 2011), information about the potential effect of environmental pollutants of this form of implicit learning is still missing. Thus, the development of medium- and high-throughput assays suitable for identifying environmental pollutants altering escape response and habituation in fish larvae is urgently needed.

Zebrafish is a cyprinid increasingly used as a vertebrate model for the study of the molecular mechanisms of brain function (Babin et al., 2014; Faria et al., 2017; Gómez-Canela et al., 2018), with the key advantage of being suitable for in vivo high-throughput screening of chemical libraries for pharmacological and/or toxicological effects. An assay to assess short-term habituation in zebrafish larvae, based on determining the motor activity of the larvae after the delivery of repetitive acoustic stimuli, was recently developed (Best et al., 2008). By using this assay, the modulation of the C-startle and habituation by different cognitive enhancers has been demonstrated. However, the fact that the above mentioned assay used a homemade setup for video-recording and the delivery of the acoustic stimuli, makes it difficult to implement in other labs and to compare results among different labs.

In this study, a new high-throughput assay for identifying compounds able to impair the vibrational C-startle response and the short-term habituation has been developed in zebrafish larvae. The vibrational startle response assay (VSRA) is based on measuring the distance moved by each larva in response to repetitive vibrational stimuli generated by a tapping device on a 48-wells microplate. Although VSRA has been developed using a commercial platform for automatizing the stimuli delivery, videotracking and further data analysis, it can be easily adapted to other existing zebrafish platforms. The first step after developing VSRA was to determine if the progressive reduction observed in the motor response after repeated stimulation met the main criteria established for habituation (Best et al., 2008; Brown, 1998; Thompson and Spencer, 1966). Then, VSRA was used to determine startle and habituation in a battery of 10 neuroactive compounds modulating cholinergic, serotonergic and glutamatergic systems, in order to analyze the concordance of VSRA with the existing data in fish and rodents (Table S1) (Leussis and Bolivar, 2006). Finally, the developed assay was used to analyze the effect of chlorpyrifos oxon and imidacloprid, as a proof of concept of the applicability of this assay to test environmental pollutants (Table S1).

2. Methods

2.1. Fish husbandry and larvae production

Adult wild-type zebrafish, purchased from Piscicultura Superior SL, Parets del Vallès, Barcelona, were maintained in fish water [reverse-osmosis purified water containing 90 μg/mL of Instant Ocean (Aquarium Systems, Sarrebourg, France) and 0.58 mM $CaSO_4 \cdot 2H_2O$] at 28 ± 1 °C in the Research and Development Centre of the Spanish Research Council (CID-CSIC) facilities under standard conditions. Embryos were obtained by natural mating and maintained in fish water at 28.5 °C on a 12 light:12 dark photoperiod. Larvae were not fed during the experimental period. All procedures were approved by the Institutional Animal Care and Use Committees at the CID-CSIC and conducted in accordance with the institutional guidelines under a license from the local government (agreement number 9027).

2.2. Experimental procedure

The chemicals used for this study were of certified laboratory high quality grade and can be found enlisted in the Supplementary material document under Section S1.1 of Supplementary methods. Stock solutions of nicotine, pilocarpine, buspirone, chloro DL phenylalanine (PCPA), deprenyl donezepil, imidacloprid, chlorpyrifos oxon (CPO) and Methyllycaconitine (MLA) were prepared in DMSO on the day of the experiment. Whereas experimental solutions for these compounds were prepared in fish water from the stock solutions, those for memantine, fluoxetine and scopolamine were directly prepared in fish water. The final concentration of DMSO in all the exposure solutions was 0.1%, except for scopolamine. As this compound exhibits very low permeability in zebrafish larvae, DMSO concentration 1% in order to increase the permeability. Solvent controls containing 0.1% or 1% DMSO were used.

Zebrafish larvae were treated with selected compounds for 24 h from 7 to 8 dpf (days post fertilization). Experiments were conducted in 48 well plates with 1 larva per well and 1 mL of medium. Plates were placed in a POL-EKO APARATURA Climatic chamber KK350 (Poland) at 28.5 °C and 12L:12D photoperiod. Larvae were never fed throughout the experimental period.

At least two independent experiments were preformed where groups of 48 larvae underwent behavioral testing. Compounds were initially evaluated for toxicity before habituation testing. Briefly, toxicity was ascertained in 8 dpf zebrafish larvae after 24 h of exposure and was established either by death, gross morphology and/or swimming impairment or clear decrease in the escape response evoked by the tapping on the plate. The highest concentration, which did not induce toxicity, was used in the subsequent VSR assay.

2.3. Vibrational startle response assay (VSRA)

The vibrational startle response assay (VSRA) was based in the automatized delivery of the vibrational stimuli using the DanioVision Tapping Device DVTD-0010, installed in a DanioVision Observation Chamber (DVOC-0040). Videotracking and the escape response were analyzed using the EthoVision XT 9 software (Noldus, Wageningen, The Netherlands) (Fig. S1 – Supplementary material). A DanioVision Temperature Control Unit (DVTCU-0011) guaranteed that all trials were performed at 28 °C. All, tapping stimulus was selected at the highest intensity (intensity level: 8), and then, sequences of the vibrational stimuli were delivered during fixed time periods referred to as interstimulus interval (ISI). Trails were conducted in 48 well plates, with one 8 dpf zebrafish larvae in each well containing 1 mL of exposure medium. Before delivering the first stimulus, larvae were left in the DVOC for 30 min to acclimate. Videos were recorded at 30 frames per second and the VSR was analyzed for each individual larva by measuring the distance moved (cm) over the 1 s period after stimulus.

2.4. Assessment of habituation criteria

In order to determine if a sequence of vibrational stimulus is able to induce a true habituation, different criteria were evaluated. Briefly, 8 dpf zebrafish larvae were placed individually in 48 well plates containing 1 mL of fish water medium. For induction of habituation, tapping stimulus was delivered at 1 s, 5 s or 20 s ISI at sessions that lasted up to 100 taps. Subsequently, the recovery of tapping startle was elicited by submitting 48 larvae to two series of tapping sessions (1 s ISI) separated by 15 min. Finally, to confirm that the VSR attenuation was indeed habituation and not the result of sensory adaptation or motor fatigue, the effect of a cross-modal stimulation was tested. Thus, larvae were subjected to a 5 s ISI regime and a 2 s white light pulse, at a white light intensity of 200 lx (5% intensity level in DanioVision settings) was delivered after the 80th tapping, recording larvae movement for the remaining 20 tapings. To confirm dishabituation, larvae were subjected to a 5 s ISI regime and a 2 s white light pulse was introduced after the 80th tapping (400 s), recording larvae movement for the remaining 100 s (time interval equivalent to 81–100th tapping) with no further vibrational or visual stimuli.

2.5. Concordance analysis

For the concordance analysis, 7 dpf larvae were exposed for 24 h to a battery of pharmacologically active compounds added to fish water medium after which were then placed in the DVOC system for tracking and analysis. Startle responses were evoked in 8 dpf control (DMSO) and treated larvae ($n = 96–240$ larvae per condition from two independent experiments) by producing a series of vibrating stimulus at 1 s ISI, for a total of 50 stimuli, synchronized by EthoVision software.

2.6. Data analysis

Data were analyzed with IBM SPSS 19.0 (Statistical Package 2010, Chicago, IL), using Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparison test. The statistical test used for each set of results can be found in the text or in the figure caption. For further clarification, Student's *t*-test analysis comparing mean distance moved of treated with that of the paired control larvae, was used for each point of plots of larvae movement during stimuli delivery trials. Data are presented as the mean ± SEM of 2 independent experiments, unless otherwise stated. Significance was set at $P < 0.05$. The area under the curve (AUC) was calculated using $Y = 0$. To simplify data representation, responses (distance moved) from the first 20 stimuli are represented in graphs plotting the VSR of each neurotransmitter modulator, while the results representing the AUC were calculated for all 50 stimulus.

3. Results and discussion

3.1. Escape response and habituation can be determined in the VSR assay

The VSRA uses zebrafish larvae instead of embryos. Although zebrafish is used at unlicensed stages for developmental neurotoxicity studies, we decided to select 7–8 dpf larvae for the development of the assay because, while they are still suitable for high-throughput screening of chemical libraries, the potential confounding factor of neurodevelopmental processes is strongly reduced at this developmental stage. In fact, by 7–8 dpf neuronal proliferation is limited to only a few particular regions, with most regions of the brain comprised of post-mitotic neurons with well-elaborated neuronal arbors (Fero et al., 2011). Moreover, complex behaviors such as responses to visual and acoustic/vibrational stimuli are only apparent in larvae (Fleming, 2007).

Response decrements resulting from repeated stimulation can be produced not only by habituation, but also by other mechanisms including receptor adaptation and effector fatigue (Thompson, 2009). Thus, any method proposed for habituation analysis needs to meet a number of hallmark criteria (Best et al., 2008; Thompson and Spencer, 1966) including: (1) the more rapid the frequency of stimulation the more rapid and/or more pronounced is habituation; (2) habituated responses exhibit spontaneous recovery and (3) instant recovery from habituation by delivery of a second stimulus, also called dishabituation. To test the first criteria of habituation, VSR was analyzed in 8 dpf zebrafish larvae using different ISIs (1 s, 5 s and 20 s), for a total of 50 tapping stimulus (Fig. 1A). In all groups, the presentation of the first tapping stimulus dramatically evoked escape response (see Video 1 – Supplementary material), determined here as the distance moved during 1 s after stimulus (Student's *t*-test: $P < 0.001$). Although the distance moved during the first startle response was similar across the different ISIs, ranging around 0.7 cm (one-way ANOVA: $F_{(2,128)} = 1.443$; $P > 0.05$), AUC increased significantly with the interval of the stimulus (one-way ANOVA: $F_{(2,149)} = 129.742$; $P < 0.001$). Thus, AUCs reached for 1 s, 5 s and 20 s ISIs were 6.16 ± 0.86, 15.42 ± 1.50 and 20.25 ± 1.76, respectively. At the shortest interval of stimuli used (ISI: 1 s) larvae responses decreased to baseline levels (pre-trial phase) by the 14th tap. For the 5 s ISI regime, larvae responses reached a steady state by the 8th tap, although this new established baseline was 4 fold higher than the 1 s ISI routine. On the other hand, during the 20s ISI regime, larvae movement following the 1st tap remained relatively high and varied between 0.5 and 0.22 cm, throughout the rest of the trial. For testing the second criteria for habituation, spontaneous recovery, larvae were tested with two series of 20 tapping stimulus delivered at 1 s ISI rate separated by a period of 15 min (Fig. 1B). Whereas an iterative reduction in startle response was found across the first series of tapping stimulus, reaching the steady state by the 19th tap ($P = 0.237$), the startle response was fully recovered in the second series of tapping stimulation. The increase in the larvae movement after the first tap was similar in both series (0.640 ± 0.036 cm vs 0.570 ± 0.031 cm for the first and second series of tapping stimulation, respectively), and the magnitude of the movement across both series was within the same range (Student's *t*-test: $P > 0.05$). Finally, in order to assure that the iterative decrease in the response observed in VSRA corresponds to true habituation and not to fatigue nor sensory adaptation (Thompson and Spencer, 1966), dishabituation of the larvae was analyzed. For inducing dishabituation, a different type of stimulus, a 2 s white-light pulse, was used. A series of 100 tapping stimulus delivered at 5 s ISI was used to cause habituation. Four trial setup combinations (Fig. 1C–F) of tapping and white-light pulse were used to establish dishabituation of habituated larvae. As previously demonstrated for 5 s ISI routine, larvae movement increased significantly in response to the first tap and then, an iterative reduction in the responses was observed until the 8–9th tap, when motor activity reached a steady state (Fig. 1C). However, when a 2 s white-light pulse was introduced after the 80th tap, the 81st tap evoked an

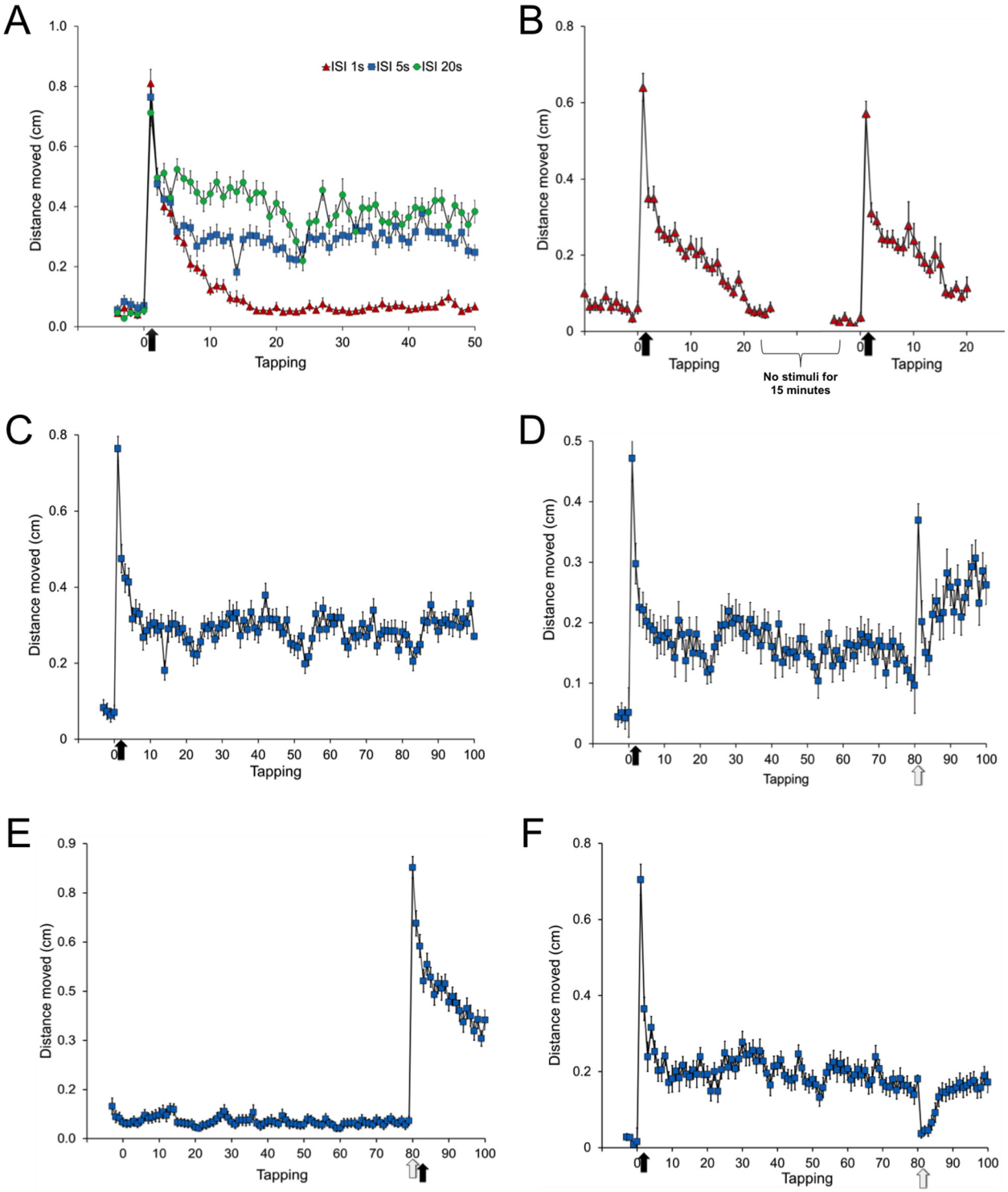

Fig. 1. Vibrational startle response assay (VSRA) meets the main habituation criteria. Plots of results of mean distance moved ± SE (n = 48) against tapping stimulus in zebrafish larvae 8 dpf. Black arrow indicates the beginning of tapping stimuli. (A) Results of 50 tapping stimulus delivered at three different interstimulus interval (ISI): 1 s (red triangle), 5 s (blue squares) and 20s (green circles); (B) larvae show a spontaneous recovery of startle response from a first session of 20 tapping stimulus at 1 s ISI (solid black line, black dots) after 15 minute period (straight brackets). The spontaneous recovery is observed when larvae undergo a second session of 20 tapping stimulus of 1 s ISI (dashed black line, black dots); (C–F) Cross-modal stimulation. Plotted results of mean distance moved ± SE (n = 48) against tapping stimulus under a 5 s ISI regime with a total of 100 stimuli. Black arrow indicates the beginning of tapping stimulus. White arrow indicates 2 s light pulse (5% intensity, according to DanioVision equipment). (C) 100 tapping stimulus; (D) 100 tapping stimulus with two second light pulse interposed between the 80th and 81st tapping; (E) no stimuli given for an equivalent time to 80 tapping stimulus (5 s ISI, total time of 400 s) followed by a two second light pulse, followed by the first (corresponding to tapping stimulus 81th) of 19 consecutive tapping stimulus; (F) 80 tapping stimulus followed by two second light pulse and no further tapping stimulus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

escape response with a similar magnitude to that of the first tap ($P > 0.05$). Interestingly, escape response remained elevated for the rest of the trial following the startle restart (Fig. 1D). On the other hand, the white-light pulse by itself did not evoke an escape response. In fact, when no more tapping stimuli were delivered after the 2 s light pulse, larvae movement significantly decreased ($P < 0.001$) at the time

corresponding to the 81st tapping stimulus and then, locomotor activity increased gradually, returning to pre-light pulse levels at the time corresponding to the 90th tapping stimulus (Fig. 1F). The results presented above demonstrate that the proposed assay not only allows quantifying the distance moved for each larva during the VSR, but also meets the criteria proposed for habituation analysis.

3.2. Concordance analysis of VSR assay

An important criterion for the validation of VSRA is that whereas the assay is performed on zebrafish larvae, results should be predictive of the responses in other vertebrate species, including other fish species and mammals. In order to assess the VSRA predictivity, the concordance

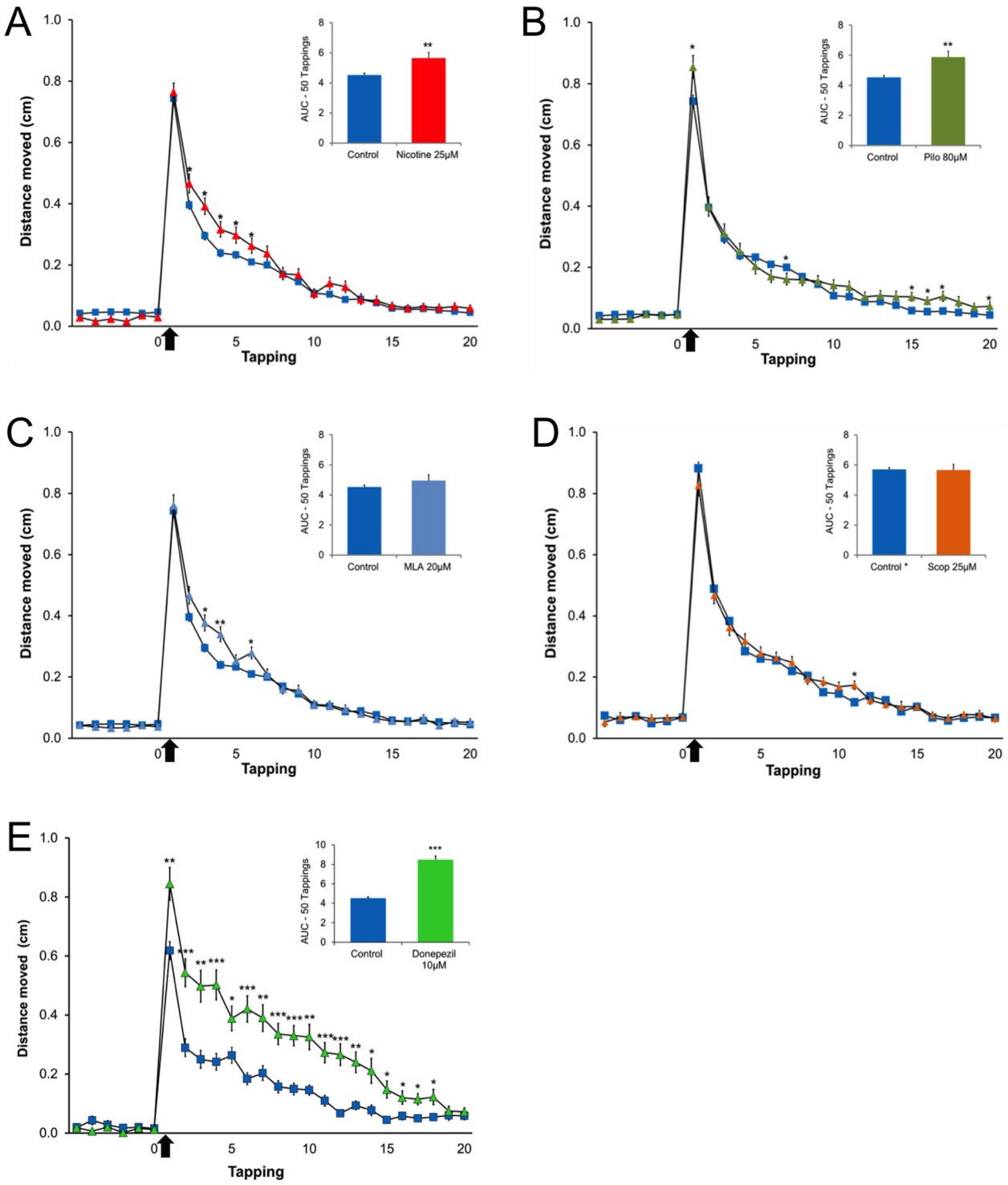

Fig. 2. Effect of cholinergic modulators on the visual startle response and habituation. Plots of average distance moved ± SE against 20 tapping stimulus and corresponding bar graphs of calculated AUC (mean ± SE) of 50 tapping stimuli at 1 s ISI. The results presented refer to control larvae (blue squares/bar) and larvae exposed to compounds that modulate the cholinergic system: (A) – 25 μM nicotine (red triangles/bar) (n = 89/point); (B) – 80 μM pilocarpine (Pilo; olive green triangles/bar) (n = 88/point); (C) – 20 μM methyllcaconitine (MLA; light blue triangles/bar) (n = 86/point) (D) – 25 μM scopolamine (Scop; orange triangles/bar) (n = 86/point); and (E) 10 μM of donepezil (green triangles/bar) (n = 48/point). Each point of control DMSO 0.1 and 1% (*) corresponds to n = 328 and n = 91 respectively. Black arrow indicates the beginning of tapping stimulus trial. ***P < 0.001; **P < 0.01 and *P < 0.05 vs corresponding control value (Student's *t*-test) of plots and bar graphs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of the results obtained with this assay with preexisting data in fish and mammals has been analyzed. For this study of concordance analysis, a group of neuroactive compounds modulating three different neurotransmitter systems have been selected (Best et al., 2008; Roberts et al., 2013; Roberts et al., 2011; Wolman et al., 2011).

3.2.1. Cholinergic system

The cholinergic system plays a pivotal role in learning and memory (Robinson et al., 2011), with cholinergic agonists improving memory (Mattson, 2004). In the present study, the effects of 25 μM nicotine, 20 μM MLA, 80 μM pilocarpine, 25 μM scopolamine and 10 μM donepezil on the distance moved during the escape response and the habituation to VSR have been analyzed using the VSRA (Fig. 2). Nicotine and pilocarpine, agonists of nicotinic and muscarinic acetylcholine receptors (AChR), respectively, significantly reduced habituation of VSR, as indicated by the significant increase in the AUC values found in treated larvae respect to the control (Fig. 2A,B). A detailed analysis of the responses across the assay showed that the nicotine effect was restricted to the responses elicited by the 2nd to 6th tapping stimuli ($P < 0.05$; Fig. 3A). Pilocarpine increased significantly the magnitude of the first startle response ($P = 0.005$; Fig. 2B) as well as the responses elicited from the 15th tapping stimulus onwards. In contrast to the effect of cholinergic agonists, no clear effects of MLA and scopolamine, antagonists of nicotinic and muscarinic AChRs, respectively, on the VSR magnitude and habituation were found (Fig. 2C,D), as indicated by the similar values of the AUC between treated larvae respect to the control (Fig. 2C,D; $P > 0.05$). Finally, the effect of donepezil, an acetylcholinesterase (AChE) inhibitor used as cognitive enhancer in Alzheimer's disease patients, was consistent with its AChRs agonist role (Fig. 2E,E). On one hand, donepezil induced a significant increase in the magnitude of the first startle response ($P < 0.001$). Moreover, donepezil significantly reduced habituation to VSR, as indicated by the significant increase observed in the AUC values (Fig. 2E) as well as in the higher response found after all the vibrational stimuli delivered in the assay (Fig. 2E).

These results with the modulators of the cholinergic system obtained by using the developed VSR assay are consistent with those reported in the literature. Thus, a significant increase of magnitude of the startle response evoked by acoustic/vibrational stimulus has been reported in rodents and zebrafish exposed to nicotine and pilocarpine (Acri et al., 1991; Acri et al., 1994; Eddins et al., 2010; Kumari et al., 2001; Schreiber et al., 2002). Moreover, the effect of 10 μM donepezil on the startle response evoked by acoustic stimulation on 7 dpf zebrafish larvae has been recently characterized (Best et al., 2008). Consistent with the results obtained in this study using vibrational stimuli, donepezil enhanced acoustic startle response and significantly reduced habituation. In the same study, authors did not find any effect on habituation using MLA and atropine (muscarinic AChR antagonist), a result also consistent with that obtained using the VSRA. Finally, scopolamine failed to induce any significant effect on the VSR magnitude and

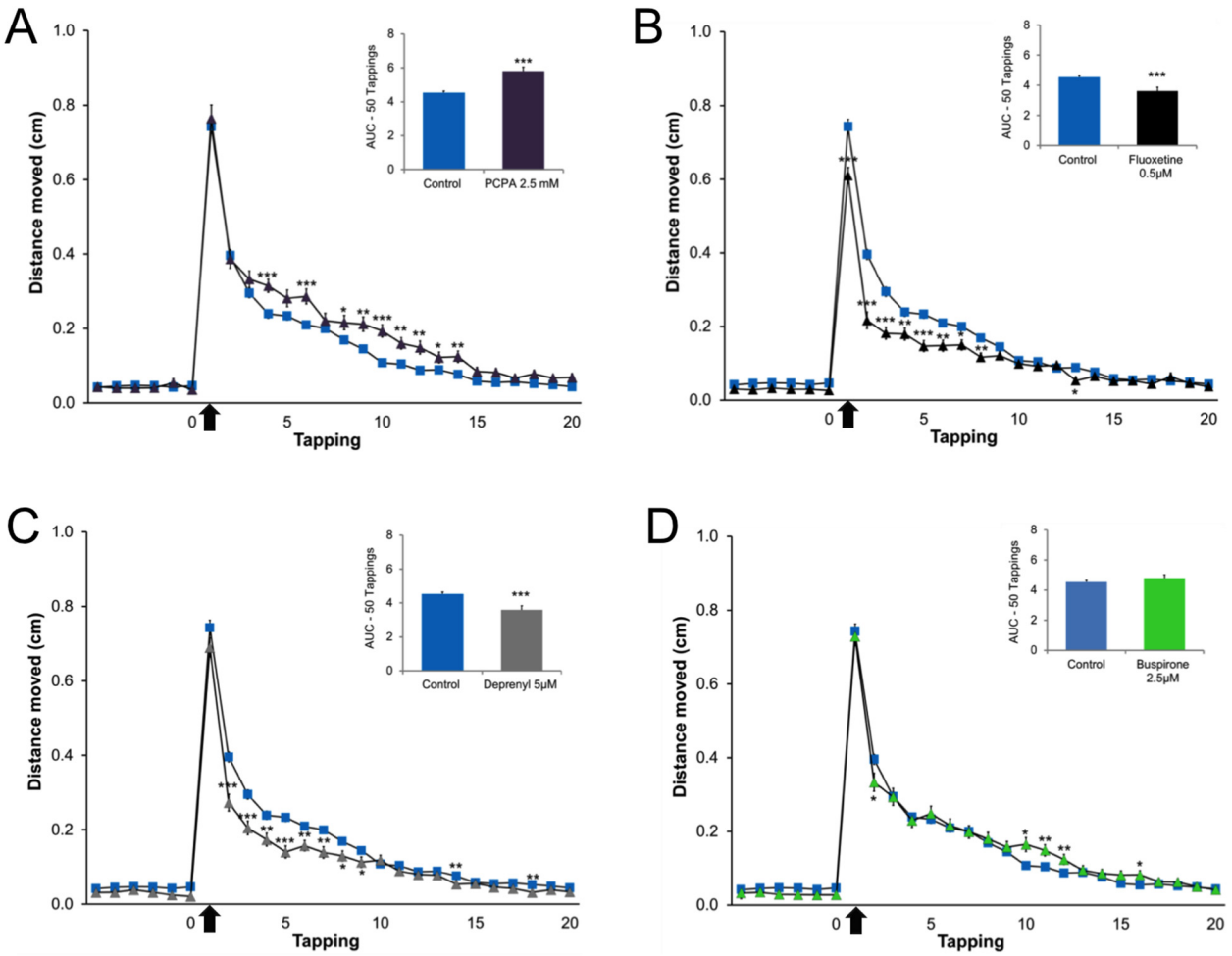

Fig. 3. Effect of serotonergic modulators on the visual startle response and habituation. Plots of average distance moved ± SE against 20 tapping stimulus and corresponding bar graphs of calculated AUC (mean ± SE) of 50 tapping stimuli at 1 s ISI. The results presented refer to control larvae (blue squares/bar, n = 328) and larvae exposed to compounds that modulate the serotonergic system: (A) 2.5 mM PCPA (dark purple triangles/bar, n = 94); (B) 0.5 μM fluoxetine (black triangles/bar, n = 93); (C) 5 μM deprenyl (grey triangles/bar, n = 94) and (D) 2.5 μM buspirone (green triangles/bar, n = 91). Black arrow indicates the beginning of tapping stimuli trial. ***$P < 0.001$; **$P < 0.01$ and *$P < 0.05$ vs corresponding control value (Student's *t*-test) of plots and bar graphs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

habituation in adult zebrafish (Levin and Cerutti, 2009), a result also consistent with the results presented in this study using VSRA.

3.2.2. Serotonergic system

Serotonin (5 HT) is a monoamine neurotransmitter involved in the control of mood, cognition and memory. Serotonergic neurons are known to modulate responses of the organism to environmental stimulation (Conner et al., 1970; Pittman and Lott, 2014; Quednow et al., 2004). To determine the effect of well-known modulators of the serotonergic system on the VSR magnitude and habituation, larvae were incubated for 24 h with 2.5 mM PCPA (tryptophan 5 hydroxylase inhibitor), 0.5 μM fluoxetine (selective serotonin reuptake inhibitor), 5 μM deprenyl (monoamine oxidase inhibitor) and 2.5 μM buspirone (5 HT1A receptor agonist; Fig. 3). Changes in the escape response of the larvae were evaluated during 50 consecutive tapping stimulus delivered at 1 s ISI rate. Decreasing serotonin synthesis with PCPA significantly reduced habituation of VSR, as indicated for the significant increase in the AUC values found in the treated larvae respect to the control ($P < 0.001$; Fig. 3A). Interestingly, the modulatory effect of PCPA on habituation was restricted to the responses elicited between the 4nd and 14th tapping stimulus ($P < 0.001$; Fig. 3A). The effect of PCPA in the VSRA is consistent with the reduced habituation of ASR found in rats exposed to PCPA (Carlton and Advokat, 1973; Conner et al., 1970; File, 1977). In contrast to the effects of the tryptophan hydroxylase inhibitor PCPA, increasing of serotonin levels at the serotonergic synapses with fluoxetine and deprenyl significantly increased habituation to VSR, specially reducing the escape responses to the first 8–10 tapping stimulus (Fig. 3B,C). This increase in habituation found after treatment with fluoxetine and deprenyl was confirmed by the significant decrease found in the AUCs when compared to control ($P < 0.001$; Fig. 3B,C). Interestingly, only fluoxetine was able to reduce VSR magnitude after the first tapping stimulus ($P < 0.001$). Results of fluoxetine and deprenyl in the VSRA are also consistent with the reported role of serotonin promoting habituation of the startle response by enhancing glycinergic inhibition of the Mauthner cells (Fero et al., 2011; Mintz et al., 1989). No clear effects were found in the VSR magnitude or habituation with the 5 HT1A receptor agonist buspirone. This absence of modulatory effect of buspirone might be related to the fact that 5 HT5 and 5HT6, but not 5 HT1, are expressed in Mauthner cells (Whitaker et al., 2011).

3.2.3. Glutamatergic system

Glutamatergic system also plays an important role in learning and memory in vertebrates. When larvae were incubated for 24 h with 40 μM memantine, an NMDA receptor antagonist, and recorded during 50 consecutive tapping stimulus delivered at 1 s ISI rate, a significant reduction in the habituation of VSR was found ($P < 0.05$), as indicated by a significant increase of the AUC of treated larvae ($P < 0.001$; Fig. 4). Additionally, response to the first tap in memantine-treated larvae was higher than in controls, and the response remained higher during the following 3 stimulus (Fig. 4). Consistent with the results obtained with VSRA in this study, previous reports on the effect of the NMDA receptor antagonists on the ASR in mice and zebrafish showed also reduced habituation compared to controls (Best et al., 2008; Klamer et al., 2004). Three different NMDA receptor antagonists also reduced short-term habituation in response to vibrational stimuli in zebrafish larvae (Wolman et al., 2011). Moreover, the higher magnitude of the escape response after the first tap found with the VSRA is also consistent with the reported effects on the ASR in larval zebrafish exposed to 30 μM memantine (Best et al., 2008).

3.2.4. Environmentally relevant compounds

The last part of this work was to assess the suitability of VSRA for dealing with environmental relevant compounds. Imidacloprid and chlorpyrifos oxon (CPO), two neurotoxic pesticides disrupting normal cholinergic signaling, were selected as a proof of concept. Imidacloprid belongs to the group of nicotine-related insecticides referred to as

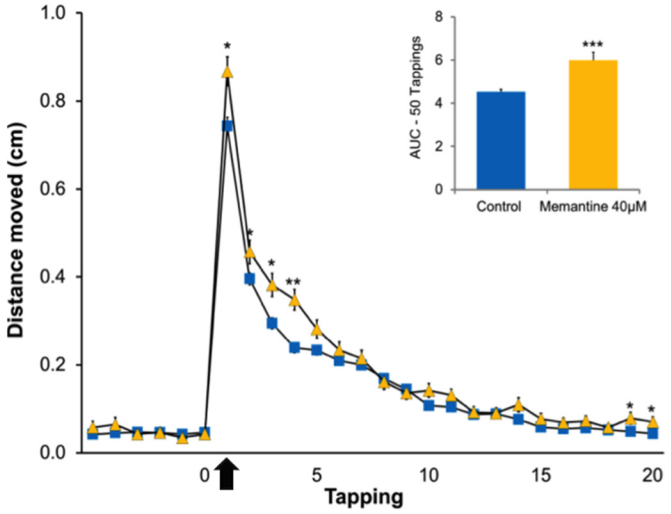

Fig. 4. Effect of memantine, a NMDA receptor antagonist, on the vibrational startle response and habituation. Plot of average distance moved ± SE against 20 tapping stimulus at 1 s ISI for control larvae (blue squares; n = 328) and larvae exposed to 40 μM memantine (dark yellow triangles; n = 91/point). Black arrow indicates the beginning of tapping stimulus trial. Inset: AUC (mean ± SE) corresponding to 50 tapping stimulus at 1 s ISI of 40 μM memantine and control. ***$P < 0.001$; **$P < 0.01$ and *$P < 0.05$ vs corresponding control value (Student's *t*-test) of the plots and bar graphs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

neonicotinoids, which act as agonists of the insect postsynaptic nicotinic acetylcholine receptors (nAChRs) (Matsuda et al., 2001) resulting in the impairment of normal nerve function. CPO, the active metabolite of chlorpyrifos, is a prototypic organophosphate (OP) with a toxic underlying mechanism that involves the irreversible inhibition of acetylcholinesterase (AChE) and overstimulation of both AChRs (Faria et al., 2015). To test the suitability of VSRA to assess quantitative concentration-response relationship, zebrafish larvae were exposed to three concentrations of each pesticide for 24 h. We found that both contaminants disrupted normal VSR magnitude and habituation in a dose dependent matter (Figs. 5 and 6). Likewise, 25 and 50 μM of imidacloprid decreased the VSR magnitude and increased habituation (Fig. 5B–D) to similar levels ($P < 0.05$), and no effect was observed at 5 μM ($P = 0.174$; Fig. 5A,D). Despite this, analysis of AUC reported significant effects across concentrations (one-way ANOVA: $F_{(3,186)} = 14.280$; $P < 0.001$), (Fig. 5D), which indicates that concentration of imidacloprid had a significant effect over VSR response. Interestingly, these results are not within the same line as that observed for nicotine, another nAChR agonist. Although the effects of imidacloprid on habituation have been well-established in honeybee (Decourtye et al., 2004; Goñalons and Farina, 2018), the effects of this compound in fish have not been well characterized.

In the only available report addressing the effect of imidacloprid on startle response and habituation in fish, an increase in the VSR magnitude and a decrease in habituation was found in adolescent zebrafish exposed to 45–60 μM imidacloprid during the first 5 days of development (Crosby et al., 2015). The differences found between that study and our results using VSRA could be related with the different experimental design used, including different exposure time, developmental stage at the time of exposure and time between the end of exposure to the behavioral testing.

Chlorpyrifos oxon evoked a dramatic dose dependent impairment of the VSR in larvae after 24 h of exposure (one-way ANOVA: $F_{(3,182)} = 35.163$, $P < 0.001$). VSR magnitude and habituation were respectively higher and lower than control (Fig. 6A–D). After the first tap, VSR magnitude in larvae treated with 25 and 50 nM CPO were already 1.4- and 3-fold higher than the corresponding controls, respectively, and remained significantly elevated throughout most of the first 20 stimuli. On the

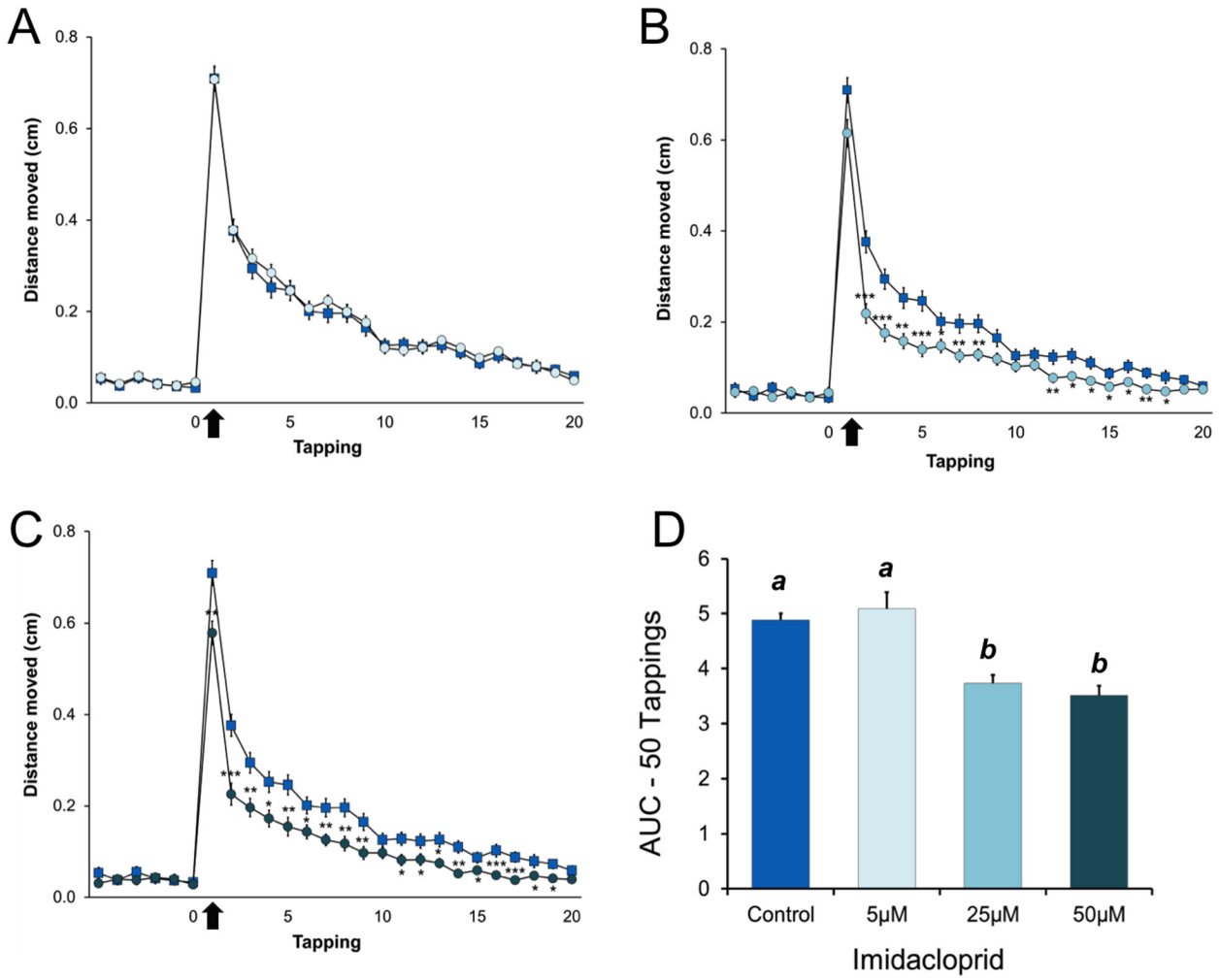

Fig. 5. Effects of imidacloprid on the vibrational startle response and habituation. (A–C) Plots of average distance moved ± SE against 20 tapping stimulus at 1 s ISI for control larvae (blue squares; n = 96) and larvae exposed to increasing concentrations of imidacloprid (circles with different shades of marine blue): 5 μM (A; n = 47/point), 25 μM (B; n = 94/point) and 50 μM (C; n = 89/point). Black arrow indicates the beginning of tapping stimuli trial; (D) AUC (mean ± SE) corresponding to 50 tapping stimulus at 1 s ISI of control and 5, 25 and 50 μM imidacloprid. The following symbols represent statistic differences for plots: ***P < 0.001; **P < 0.01 and *P < 0.05 vs corresponding control value, Student's *t*-test. For the bar graph, different letters indicate significant (P < 0.05) differences following one-way ANOVA and Tukey's multiple-comparison test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

other hand, 5 nM CPO seemed to have a delayed effect over VSR magnitude, presenting significant differences from the 6–14th tapping stimuli. Eventually, habituation of the VSR was reached for all treatments before the 20th tapping stimulus. AUC values were already notably significant at 5 nM (P = 0.014) (Fig. 6D). Impairment of the startle response by chlorpyrifos has been reported in different fish species and rodents (Carlson et al., 1998; Eddins et al., 2010; Levin et al., 2002; Sledge et al., 2011). Additionally, these responses, though much more potent, resemble those reported for the two AChR agonists and for the cholinergic drug tested in this study, which emphasizes the robustness of the proposed method and reinforces this approach as a promising behavioral monitoring tool for screening and differentiating chemicals present in the environment.

Zebrafish larvae are ideally suited for large-scale analysis of vertebrate behavior, including learning and memory (Colwill Ruth and Creton, 2011; Levin and Chen, 2004). For medium- to high-throughput analyses of behavior, it is crucial to use robust assays that can be automated. Indeed, easy to use automated systems, designed as a plug and play system, including both software and equipment, have been recently developed and are currently commercially available for imaging and analyzing zebrafish larvae behavior in multi-well plates. However, to our knowledge, implicit learning evaluation methods have not yet been validated for any commercially available platform. In this study we have demonstrated that an automated system using vibrational stimulation was able to fulfill a number of criteria that have been established to determine true habituation (Brown, 1998; Thompson, 2009; Thompson and Spencer, 1966). In this study, zebrafish larvae demonstrated the basic response of habituation, an iterative reduction of the distance moved in response to repetitive vibrational stimuli, fulfilling the main criteria proposed for habituation. Aiming to analyze the predictivity of the results of this assay performed with zebrafish larvae to other vertebrate species inhabiting aquatic ecosystems, a group of modulators of the cholinergic, serotonergic and glutamatergic systems was tested, and the results were in agreement with the data available in the bibliography for fish and rodents. This concordance of the results in the developed assay with others developed in the same and different vertebrate species demonstrate the results from this assay can be easily extrapolated to other aquatic vertebrates. The results regarding CPO and imidacloprid also demonstrated that the developed assay is suitable for the analysis of the concentration-response relationship of environmental pollutants that impair the escape response elicited by vibrational stimuli. This result is highly relevant, since the concentration-response relationships obtained in this assay might provide an estimation of the potency (EC50 or EC10) of the disrupting effect of each tested pollutant on the predator avoidance behavior. Moreover, the combination of concentration-response data from VSRA with data

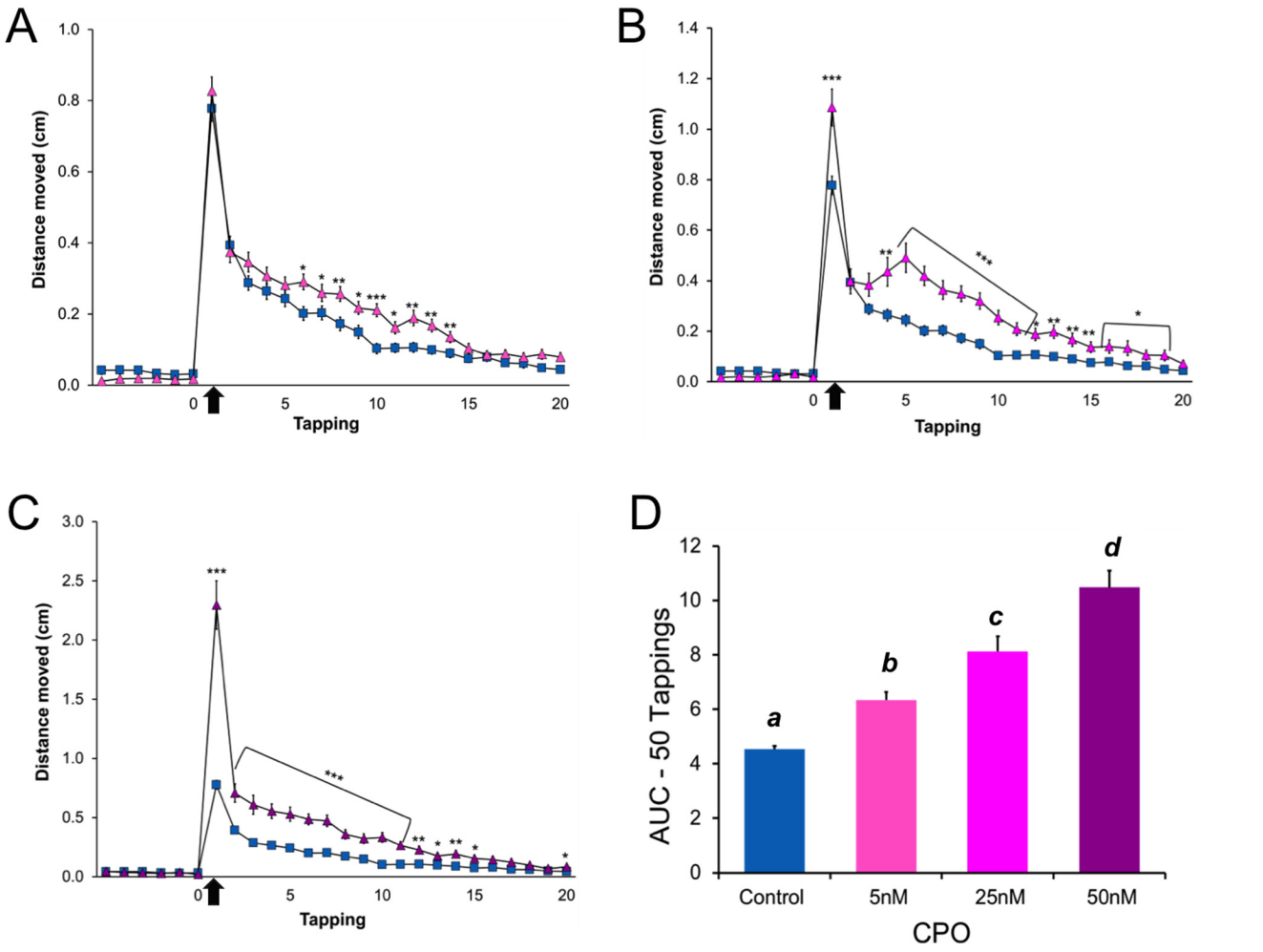

Fig. 6. Effects of chlorpyrifos oxon (CPO) on the vibrational startle response and habituation. (A–C) Plots of average distance moved ± SE against 20 tapping stimulus at 1 s ISI for control larvae (blue squares; n = 96) and larvae exposed to increasing concentrations of CPO (triangles with different shades of pink): 5 nM (A; n = 88/point), 25 nM (B; n = 86/point) and 50 nM (C; n = 88/point). Black arrow indicates the beginning of tapping stimulus; (D) AUC (mean ± SE) corresponding to 50 tapping stimulus at 1 s ISI of control and 5, 25 and 50 nM CPO. The following symbols represent statistic differences for plots: ***P < 0.001; **P < 0.01 and *P < 0.05 vs corresponding control value, Student's *t*-test. For the bar graph, different letters indicate significant (P < 0.05) differences following one-way ANOVA and Tukey's multiple-comparison test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

about lethality should allow to determine the associated hazard (LC50/EC50) to any tested compound (Thienpont et al., 2011). Additional efforts should be done to assess the suitability of this new assay to determine the potency and hazard of environmental pollutants.

4. Conclusion

We have developed a new simple and automated assay in zebrafish larvae, for in vivo medium to high-throughput assessment of the escape response and its habituation. We were able to demonstrate the assay's predictive and feasible qualities for the potential development of a systematic approach able to screen chemical compounds present in the environment with specific effects over fish escape behavior, as well as, establish fish species more sensitive or resistant to such chemicals.

List of the neuroactive compounds and environmental contaminants used for this study. Supplementary data to this article can be found online at doi:https://doi.org/10.1016/j.scitotenv.2018.08.421.

Author contributions

M.F and E.P performed all the exposure experiments; M.F, C.G-C, K.A. N-L and J.B performed the behavioral analyses; D.R. was involved in the conception; M.F and D.R were involved in the design and interpretation of the data and the writing of the manuscript with input from L.M.G-O.

Competing financial interests

The authors declare no competing financial interest.

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