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Heart rate and behavioral responses in three phylogenetically distant aquatic model organisms exposed to environmental concentrations of carbaryl and fenitrothion

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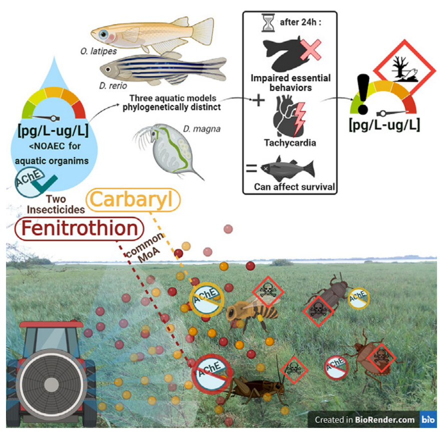
HIGHLIGHTS

- Zebrafish, medaka and *D. magna* were exposed to carbaryl and fenitrothion.
- Environmental concentrations of both pesticides are below its NOAECs.
- Changes in heart rate and specific behaviors were found in the three species.
- Similar effects in these three species suggest evolutionary conserved mechanisms.
- Additional endpoints, such as behavior and heart rate, should be included in ERA.

GRAPHICAL ABSTRACT



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ABSTRACT

Carbaryl and fenitrothion are two insecticides sharing a common mode of action, the inhibition of the acetylcholinesterase (AChE) activity. Their use is now regulated or banned in different countries, and the environmental levels of both compounds in aquatic ecosystems have decreased to the range of pg/L to ng/L. As these concentrations are below the non-observed-adverse-effect-concentrations (NOAEC) for AChE inhibition reported for both compounds in aquatic organisms, there is a general agreement that the current levels of these two chemicals are safe for aquatic organisms. In this study we have exposed zebrafish, Japanese medaka and *Daphnia magna* to concentrations of carbaryl and fenitrothion under their NOAECs for 24-h, and the effects on heart rate (HR), basal locomotor activity (BLA), visual motor response (VMR), startle response (SR) and its habituation have been evaluated. Both pesticides increased the HR in the three selected model organisms, although the intensity of this effect was chemical-, concentration- and organism-dependent. The exposure to both pesticides also led to a decrease in BLA and an increase in VMR in all three species, although this effect was only significant in zebrafish larvae. For SR and its habituation, the response profile was more species- and concentration-specific. The results presented in this manuscript demonstrate that concentrations of carbaryl and fenitrothion well below their respective NOAECs induce tachycardia and the impairment of ecologically relevant behaviors in phylogenetically distinct aquatic model organisms, both vertebrates and invertebrates, emphasizing the need to include this range of concentrations in the environmental risk assessment.

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1. Introduction

Carbaryl (1-naphtyl-*N*-methylcarbamate) was one of the most widely used carbamates for the control of a broad spectrum of insects (Hastings et al., 2001). Fenitrothion (*O*-*O*-dimethyl *O*-[3-methyl-4-nitrophenyl] phosphorothioate) is an organophosphorus insecticide used in agriculture and forestry to control sucking and biting pests (Environmental Health Criteria, 1992). Carbaryl and fenitrothion share a common mode of action (MoA), the inhibition of the acetylcholinesterase (AChE) activity (Faria et al., 2021, 2022). While carbaryl and fenitrothion have been two of the most widely used insecticides for the past fifty years, during the last years their use has been strongly regulated or banned in some countries, so that environmental levels of both compounds in non-directly exposed aquatic ecosystems are now usually in the ng/L range or lower. For instance, carbaryl concentrations in Pinios river (Greece) were 10–100 ng/L (Fytianos et al., 2006). In the waterways entering the Cornet Inlet Marine National Park (Australia), carbaryl was only detected in the 5 % of the water samples analyzed (limit of reporting: 3 ng/L), with an average concentration of 4 ng/L (Allinson et al., 2016). Carbaryl was detected in 8 of 457 samples from a South Florida (USA) agricultural watershed (limit of detection: 130 ng/L), with reported concentrations of 330–1300 ng/L (Wilson and Foos, 2006). For fenitrothion, the average concentration in different draining channels River delta (Spain) was in the range 137–376 ng/L, with a frequency of detection of 22–64 % (Köck et al., 2010). At Kurose river (Japon), fenitrothion concentration ranges were from not detected (<1 ng/L) to 370 ng/L (Kaonga et al., 2015). The levels of fenitorthion were under the limit of detection (41.5 ng/L) in all the freshwater samples collected in the rural area of the Todos Santos Bay Region (Brazil) (Nascimento et al., 2021). This range of concentrations is below the non-observed-adverse-effect-concentrations (NOAEC) for AChE inhibition reported in different fish species [*carbaryl*: 66 μg/L (24 h) for *Danio rerio* larvae (Faria et al., 2022), 135 μg/L (brain extracts, 1 h) for *Rachycentron canadum* (Assis et al., 2012) and *Colossoma macropomum* (Assis et al., 2010); *fenitrothion*: 30 μg/L (96 h) for *Dicentrachus labrax* juveniles (Almeida et al., 2010) and 17 μg/L (24 h) for *Danio rerio* larvae (Faria et al., 2021)]. Therefore, there is a general agreement that the current levels of these two chemicals are safe for aquatic organisms (Environmental Health Criteria, 1992; United States Environmental Protection Agency, 2012).

Currently there are many evidences demonstrating that the exposure of aquatic organisms to environmental concentrations of different pollutants, well below their NOAECs, lead to hormetic responses in different endpoints, including the heart rate and different behaviors (Agathokleous, 2022; Agathokleous et al., 2021). For instance, we recently reported increased heart rate and altered behavior in zebrafish larvae exposed for 24 h to environmental concentrations of carbaryl (Faria et al., 2022). A similar effect on behavior was found when the zebrafish larvae were exposed to environmental concentrations of fenitrothion (Faria et al., 2021). Hormesis is considered a highly-conserved evolutionary adaptive strategy of living organisms for developing resilience, and as a result, hormetic effects are expected to occur widely in aquatic organisms, independently of the taxon considered (Agathokleous, 2022). Although there is a general misconception that hormetic effects are always beneficial, it has been demonstrated that they will be beneficial or detrimental depending on the biological context (Calabrese, 2010, 2011a; Calabrese and Baldwin, 1999).

This study aims to determine if the short-term exposure to the current environmental levels of carbaryl and fenitrothion leads to altered heart rate and behavioral responses in aquatic organisms, and whether the observed changes are produced through well-conserved mechanisms throughout evolution. Therefore, we have exposed zebrafish (*Danio rerio*), Japanese medaka (*Oryzias latipes*) and water flea (*Daphnia magna*), three model organisms widely used in aquatic toxicology, to concentrations carbaryl and fenitrothion in the range of pg/L to ng/L, well under their NOAEC, and the effects on hear rate (HR), basal locomotor activity (BLA), visual motor response (VMR), startle response (SR) and its habituation have been evaluated. The results presented in the manuscript strongly support the need to include sub-NOAEC concentrations in the ecological risk assessment of the pollutants in aquatic ecosystems.

2. Material and methods

2.1. Experimental organisms and culture conditions

Wild-type zebrafish larvae were obtained at the CID-CSIC facilities following standard protocols described elsewhere (Faria et al., 2021, 2022). All research conducted with this fish species followed the institutional guidelines under a license from the local government (license n° 11336).

Japanese medaka embryos (CAB line), at early gastrula stage, were provided by Gis-Amagen (INRA, Jouy-en-Josas, France). Upon receipt at 24 h post-fertilization (hpf), alive and synchronous embryos were sorted and maintained in Petri dishes with Egg Rearing Solution (ERS; 17.11 mM NaCl, 0.4 mM KCl, 0.36 mM $CaCl_2$, and 1.36 mM $MgSO_4$; pH 7.0) in a climate chamber (Snidjers Scientific, Tilburg, the Netherlands) at 28 ± 0.3 °C, with 12 L:12D photoperiod cycle (5000 lx white light). Dissolved oxygen concentration was measured daily using a Fibox 3 fiber-optic oxygen mini-sensor (PreSensPrecision Sensor, Regensburg, Germany). The development of medaka embryos at the selected rearing temperature reached the peak of hatching between 7 and 8 days post-fertilization (dpf). Embryonic mortality rate using these rearing conditions was between 5 and 15 %. After hatching, medaka larvae were transferred to glass beakers containing 20 mL of rearing water [dechlorinated tap water mixed with osmosed water (1/2 v/v), aerated for 24 h] until the beginning of experiments, at 10 dpf stage, two or three days after hatching. All the experiments conducted with this fish species were performed at the EPOC laboratory (University of Bordeaux, France) under the authorization number APAFIS#28934.

Bulk cultures of 10 adult females of *D. magna* (clone F) were maintained in 300 mL ASTM hard synthetic water at high food ration levels, until they released their sixth brood and were then reinitiated with newborn individuals. To achieve the required number of juvenile females needed for the experimental part, several larger parthenogenic cultures of 100 individuals were initiated with third- to sixth-brood neonates (<24 h old) from bulk cultures and maintained in 1.5 L ASTM plus algae (5×10^5 cells/mL of *Chlorella vulgaris*), for 7 days until use. Culture medium was renewed three times a week, the photoperiod was set to a 16 L:8D cycle, and the temperature was set to 20 ± 1 °C (Bedrossiantz et al., 2021).

2.2. Carbaryl and fenitrothion exposures

Carbaryl (Pestanal®, analytical standard, purity 99.9 %) and Fenitrothion (Pestanal®, analytical standard, purity 95.4 %) were obtained from Sigma-Aldrich (Steinheim, Germany). Stock concentrations of carbaryl and fenitrothion were prepared in pure DMSO, with final carrier concentration of 0.1 % was used in both vehicle control and all treatment groups. The use of vehicle controls with 0.1 % DMSO is widely used for screening libraries of small chemicals in zebrafish embryos and larvae, including those screening based in behavioral endpoints (Maes et al., 2012; Vliet et al., 2017).

Four carbaryl concentrations, 0.06 ng/L, 0.66 ng/L, 6.6 ng/L and 66 ng/, were tested. These concentrations cover the lower-middle range of the levels reported in not-directly impacted aquatic ecosystems. None of the four selected concentrations resulted in systemic toxicity (impaired gross morphology or lethality) in the three model species.

Four fenitrothion concentrations, 1.7 ng/L, 17 ng/L, 170 ng/L and 1.7 μg/L, were tested in fish larvae. The highest concentrations are representative of the situation in the vicinity of sprayed areas and the lowest ones represent the situation in non-directly exposed areas. Due to the higher sensitivity of invertebrates to this insecticide, three additional fenitrothion concentrations, 1.7 pg/L, 17 pg/L, and 170 pg/L, were tested only in *D. magna*. Since the exposure to 1.7 μg/L of fenitrothion in *D. magna* resulted in the impairment of mobility (convulsions), this concentration was only used to determine the effects on the cardiac function but not on behavior. The stability of carbaryl and fenitrothion in fish water during 24-h under our experimental conditions has recently demonstrated (Faria et al., 2021, 2022).

Exposures of fish models were conducted in multi-well microplates, using 48-well plates for behavioral assays (one larva per well in 1 mL working solution), and 6-well plates for cardiac activity assessments (20 larvae per well in 10 mL working solution). Fish larvae were maintained overnight in optimal conditions, at 28 °C with 12 L:12D photoperiod before starting experiments. Behavior and cardiac activity were directly tested after 24 h of exposure in fish species (zebrafish larvae from 7 to 8 dpf and medaka larvae from 9 to 10 dpf).

Exposures in *Daphnia* were performed in 120 mL borosilicate glass bottles with 25–50 individuals per 100 mL of working solution and maintained overnight at 20 °C with 16 L:8D photoperiod cycle. Behavioral responses of single *D. magna* individual were monitored in 24-well plates with 1 mL of working solution per well.

Prior to sampling for AChE activity analyses, fish larvae used for behavioral assays were euthanized by rapid chilling, by transferring them to ice-chilled water (2–4 °C), a method in accordance with the AVMA Guidelines for Euthanasia (Leary et al., 2020). Individual fish larvae or pools of 8 daphnia were immediately frozen on dry ice and stored at −80 °C until further analysis. Samples were collected from 2 to 3 trials of the same experiment setup conducted in different days and with different batches of animals.

2.3. Cardiac activity determination

Before video recording the heart movement, 8 dpf zebrafish and 11 dpf medaka larvae were anaesthetized with MS222 (tricaine, 170 mg/L) and then positioned in a ventral or lateral view in 4 % methylcellulose. In contrast, *Daphnia* individuals were directly positioned in lateral view in methylcellulose without the need for any anesthetic agent. Analysis of cardiac activity was performed in isolated behavior rooms set at 27–28 °C for fish larvae and 20–21 °C for *D. magna*.

The cardiac activity of each zebrafish larva or daphnia was video recorded for 30 s with a GigE camera (*zebrafish and daphnia*: UI-5240CP-NIR-GL, Imaging Development Systems, Germany; *medaka*: acA1300-60gm, Basler, Germany) mounted onto a stereomicroscope (*zebrafish and daphnia*: Motic SMZ-171, Wetzlar, Germany; *medaka*: Leica MZ7.5, Leica, Nanterre, France), basically as reported by Faria et al. (2022).

Video analyses of each individual zebrafish larva and Daphnia were performed at IDAEA-CSIC, using a recently developed MATLAB algorithm (Duran-Corbera et al., 2022), whereas the analysis of the heart rate in medaka larvae was performed at UMR-EPOC laboratory using DanioScope™ software (Noldus, Wageningen, the Netherlands; see Supplementary Fig. 1 for additional information on both interfaces). No significant differences were found in the results obtained with the two software when a same video record of a zebrafish larva (Supplementary Video 1) was analyzed with both the MATLAB algorithm and the DanioScope™ software (see Supplementary Table 1).

2.4. Behavioral analysis

2.4.1. Behavioral assessments in zebrafish and medaka larvae

Behavioral assays in fish species were performed using a DanioVision system, including a DanioVision Observation Chamber (DVOC) with near-infrared light, a Temperature Control Unit (TCU) to maintain the system at 28 °C, and the EthoVision XT 14 software (Noldus, Wageningen, the Netherlands), essentially as reported elsewhere (Chiffre et al., 2016; Faria et al., 2021, 2022).

Briefly, trials were conducted in 48 well plates, fish larvae were left in the DVOC for 20 min in the dark to acclimate before running the series of vibrational and visual stimuli that make up the behavior battery. A series of 51 tapping stimuli [intensity: 8; interstimulus interval (ISI): 1 s] in the dark was followed by a 10 min recovery time, and then, by a light cycle (intensity: 100 %, duration: 10 min) and a dark cycle (intensity: 0 %, duration: 10 min).

Videos were recorded at 30 frames per second (fps) and the vibrational startle response (VSR), visual motor response (VMR) and basal locomotor activity (BLA) of each individual larva were analyzed using the multi-tracking module of EthoVision software. VSR, based in the escape response evoked in fish larvae by a vibrational stimulus, is assessed by measuring the distance moved (cm) over the 1 s period after the stimulus. As the consecutive harmless stimuli are being delivered at a short time interval (every second), due to habituation, larvae escape responses become less intense until they completely cease (Best et al., 2008). Therefore, from this data we can evaluate two parameters: (1) the startle response (SR), corresponding to the first response of maximum intensity and (2) its habituation, calculated as the area under the response curve (AUC) from the first to the fiftieth stimulus. VMR corresponds to the hyperactivity period in reaction to a sudden reduction of light intensity in the environment, and is calculated by subtracting the distance moved (cm) over the last 2 min of the light period from the distance moved (cm) over the first 2 min of dark period. BLA is defined here as the distanced traveled by the larvae during a 10 min period without stimulation in the dark.

2.4.2. Behavioral assessments in Daphnia magna

The *D. magna* clone F is characterized by a negative phototactic behavior, therefore, as same as fish species it presents an interesting response to unexpected changes in light intensity in its environment. In a previous work, we tested the suitability of using DanioVision as a high-throughput tracking system for our behavioral studies in *Daphnia*, and developed an interesting protocol to assess the daphnia escape response evoked by a light stimulus. Daphnia Photomotor Response Assay (DPRA), described by (Bedrossiantz et al., 2020), looks very similar to the Vibrational Startle Response Assay (VSRA) designed for zebrafish by (Faria et al., 2018). In fact, we can distinguish the same two phases in the response to the stimulus, whether light (LSR) or vibrational (VSR): (1) the startle, corresponding to the innate flight response, and (2) habituation, the identification of the stimulus as not dangerous and therefore an adaptation of the animal that stops trying to flee from it.

Behavioral tests were carried out with the aim to obtain results easily comparable with those obtained in fish models (Aliko et al., 2019; Yalsuyi et al., 2021a, 2021b). For this purpose, we performed the assays using a DanioVision system, including a DVOC with near-infrared light coupled to a TCU, that maintains the system at 20 °C, the temperature of the Daphnia Behavioral Room. Similarly to the fish larvae assays, the automated delivery of visible light stimuli from the DVOC was controlled by EthoVision XT 14 software (Noldus, Wageningen, the Netherlands).

In Daphnia, trials were conducted in 24-well plates, so that the size of the well did not restrict the movement of the animal. Before delivering the first stimulus, Daphnia were left in the DVOC for 5 min to acclimate. In this case, the behavioral battery consisted of different types of light stimulations whose intensity was always set at 50 %, corresponding to 290 lx. The acclimation phase was followed by a series of light flashes (flash duration: 1 s, repetitions: 30, frequency: 0.25 Hz). Then, after a recovery time of 10 min, daphnia received a single long light signal (duration: 5 min ON, 1 min OFF).

Videos were recorded at 30 fps and the light startle response (LSR), visual motor response (VMR) and basal locomotor activity (BLA) were analyzed for each individual *D. magna* using the multi-tracking module of EthoVision software. The LSR is based in the escape response evoked in individual *D. magna* by a flash of light, and is assessed by measuring the maximum distance moved (mm) over the 5 s stimulus period. From this data we can evaluate the SR and its habituation. VMR is calculated in Daphnia by subtracting the distance moved (mm) over the 5 min of the dark period from the distance moved (mm) over the next 5 min of the light period. Similar to the fish larvae assays, BLA is defined in Daphnia as the distanced traveled by this organism during a 10 min period without stimulation in the dark.

Table 1
Acetylcholinesterase (AChE) activity in Japanese medaka larvae and *Daphnia magna* control and exposed to carbaryl (66 ng/L) and fenitrothion (170 ng/L and 1.7 μg/L) for 24 h.

	Control	Carbaryl 66 ng/L	Fenitrothion	
			1.7 μg/L	170 ng/L
Medaka AChE (μmol/min/mg protein)	0.355 ± 0.060	0.364 ± 0.079	0.320 ± 0.069	–
Daphnia AChE (nmol/min/mg protein)	1.384 ± 0.216	1.586 ± 0.139	0.276 ± 0.220***	1.282 ± 0.221

*** $p < 0.001$.

2.5. Acetylcholinesterase activity

Zebrafish acetylcholinesterase (AChE) activity data was determined as described by (Faria et al., 2021, 2022). Medaka acetylcholinesterase (AChE) activity was determined as described by (Faria et al., 2015). Finally, Daphnia AChE activity was determined by a modification of the Ellman method adapted to microplate (Barata et al., 2004). Additional information is provided in Supplementary Methods.

2.6. Statistical analysis

Data were analyzed with IBM SPSS v25 (Statistical Package 2010, Chicago, IL). To assess the normality of the data we used Shapiro–Wilk tests. To determine the significance of the differences between the different treatments and the control groups (i.e., normal or non- normal distributions), we used 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). Behavioral data of the three species were first normalized as a percentage of the corresponding controls and then, the mean values for each experimental condition were plotted as a heat map using also GraphPad Prism. Data from 2 to 3 independent experiments are presented as the mean ± SD or the median and the interquartile range, unless otherwise stated.

3. Results

3.1. Environmental concentrations of carbaryl and fenitrothion are below the NOAEC for inhibition of AChE activity

NOAECs for AChE inhibition in 8 dpf zebrafish larvae exposed for 24-h to carbaryl and fenitrothion were not determined in this study, as they have been recently reported (Faria et al., 2021, 2022). These reports indicate that both NOECs are, for the larvae of this species, about 1000-times higher than the maximum concentration of each chemical used in this study. When the AChE activity was determined in Japanese medaka (Table 1), no differences were found between control and larvae exposed to 66 ng/L carbaryl or 1.7 μg/L fenitrothion were found [$F_{(2,20)} = 0.855, p = 0.440$]. Finally, when AChE activity was determined in *Daphnia magna*, a significant effect of the treatment was found [$F_{(3,24)} = 53.375, p = 8.47 \times 10^{-11}$]. The exposure to 1.7 μg/L fenitrothion leaded to a significant decrease in Daphnia AChE activity ($p = 9.84 \times 10^{-9}$). However, no differences were found between control daphnia and those exposed to 117 ng/L fenitrothion ($p = 0.692$) or 66 ng/L carbaryl ($p = 0.240$).

3.2. Changes in the heart rate in zebrafish, medaka larvae and Daphnia magna after short-term exposure to concentrations of carbaryl and fenitrothion below their NOAECs

Average heart rates in the control group were 147.45 ± 22.86 (n = 110), 129.84 ± 4.57 (n = 11) and 463 ± 18.10 (n = 35) bpm for zebrafish larvae, medaka larvae and adult *Daphnia magna*, respectively. Fig. 1A shows that, after 24-h of exposure to concentrations of carbaryl well below their NOAECs, a positive chronotropic effect on heart rate was found in the three selected model organisms, although with differences in the intensity of this response according to the concentration and the organism. Zebrafish larvae exposed to 0.066–66 ng/L carbaryl showed a significant increase in heart ratio [$H(4) = 65.05, p = 2.52 \times 10^{-13}$], reaching values 52–55 % higher than those of control larvae in the 0.6–66 ng/L range. Heart rate also increased in medaka larvae exposed to 0.6–66 ng/L carbaryl [$H(4) = 34.57, p = 5.58 \times 10^{-7}$], although the magnitude of the increase was lower than in zebrafish, with values 7–10 % higher than those in controls. A general trend to increase the heart rate was observed in *Daphnia magna* exposed to carbaryl along the whole range of concentrations, with values approximately 4–6 % above controls. However, this effect was only significant at 66 pg/L and 66 ng/L [$H(4) = 18.56, p = 0.00096$].

A significant increase in the heart rate was also observed in the three selected model organisms after a 24 h exposure to concentrations of fenitrothion below their NOAECs (Fig. 1B) and, similarly to carbaryl, differences in the intensity of this response were observed with the concentration and the organism. When zebrafish larvae were exposed to fenitrothion, a significant increase in heart rate was observed in the 17–170 ng/L range [$H(4) = 43.75, p = 7.23 \times 10^{-9}$], with values 20–25 % above controls, returning then, at 1.7 μg/L, to the control values. Heart rate also increased in medaka larvae exposed to 17–1700 ng/L carbaryl [$H(4) = 51.47, p = 1.78 \times 10^{-10}$], although the increase observed in this species, with values between 11 and 14 % higher than in controls, was less than in zebrafish. Interestingly, fenitrothion showed a robust biphasic response in *Daphnia magna* [$H(4) = 26.92, p = 0.00002$], exhibiting only a significant increase when exposed to the lowest concentration of fenitrothion, 1.7 ng/L. Since this species is highly sensitive to fenitrothion and the highest tested concentration was above the reported NOAEC for the inhibition of AChE activity in this species, the effect of fenitrothion in the 1.7–170 pg/L range was also tested. As shown in Fig. 1C, fenitrothion significantly increased *Daphnia magna* heart rate in the range between 1.7 pg/L to 1.7 ng/L range [$H(3) = 43.57, p = 1.86 \times 10^{-9}$], with an increase of 6–10 %.

3.3. Behavioral changes in zebrafish and medaka larvae and Daphnia magna after short-term exposure to concentrations of carbaryl and fenitrothion below their NOAECs

Fig. 2 summarizes as a heat map the results of the 24-h exposure to different concentrations of carbaryl and fenitrothion below their NOAECs on basal locomotor activity (BLA), visual motor response (VMR), startle response (SR) and its habituation, determined in the three model organisms selected. First of all, a general trend to decrease BLA was observed in all three species, although this effect was only significant for zebrafish larvae [$H(4) = 80.03, p = 1.72 \times 10^{-16}$ and $H(4) = 71.04, p = 1.37 \times 10^{-14}$ for carbaryl and fenitrothion, respectively]. Moreover, the exposure to fenitrothion shows a biphasic response on locomotor activity in zebrafish larvae, with a significant decrease at the three lowest concentrations tested and then returning to control values at the highest concentration.

In contrast to the effects on BLA, the main effect of carbaryl and fenitrothion on the VMR in the three species was a general trend to increase this escape response evoked by the sudden transition from light to dark environment. As in the case of BLA, the observed effect on the VMR was only significant for zebrafish larvae [$H(4) = 24.70, p = 5.77 \times 10^{-5}$ for carbaryl; $H(4) = 55.38, p = 2.70 \times 10^{-11}$ for fenitrothion] and exclusively at the highest tested concentrations. Interestingly, a similar trend was also observed with *D. magna* VMR.

For the last two behaviors examined in this study, SR and its habituation, the response profile was on the overall more species-specific. The mild increase in the SR evoked by one vibrational stimulus observed in zebrafish larvae after the exposure to the lowest concentrations of carbaryl and fenitrothion was followed by a mild decrease in this behavior at the highest concentrations [$H(4) = 94.81, p = 1.25 \times 10^{-19}$ for carbaryl and $H(4) = 52.58, p = 1.04 \times 10^{-10}$ for fenitrothion]. In medaka, however, carbaryl exposure resulted in a trend toward a mild decrease in SR over the whole range of concentrations, whereas fenitrothion had no clear effect on this behavior. SR evoked by a visual stimulus significantly increased in *D. magna* exposed to all the selected concentrations of carbaryl [$H(4) = 31.50, p = 2.42 \times 10^{-6}$]. However, no changes in this behavior were found when this organism was exposed to fenitrothion. Finally, Fig. 2 clearly shows that the effects on habituation time, determined as AUC, was also chemical-specific. When the effect of carbaryl on habituation was determined in zebrafish larvae a rebound effect was found [$H(4) = 59.92, p = 3.01 \times 10^{-12}$]. In this species, the initial decrease in the habituation time observed after exposure to 0.66 ng/L carbaryl was followed by an increase when they were exposed to 6.6 ng/L and 66 ng/L carbaryl. A different modulatory effect on zebrafish habituation was found for fenitrothion, with a general trend to decrease habituation time, although the differences were significant only for 1.7 μg/L [$H(4) = 33.61, p = 8.95 \times 10^{-7}$]. Despite no significant changes in habituation were found in medaka larvae exposed to carbaryl or fenitrothion, after the exposure to the latter chemicals a clear trend to decrease habituation time was observed, a result consistent with the effect found in zebrafish. Finally, while the results of habituation of the startle response in *D. magna* after carbaryl exposure showed a general trend of increasing habituation time, no clear effect on habituation was found when *D. magna* was exposed to fenitrothion.

4. Discussion

In this study, an increase in the heart rate has been observed in the three model organisms following 24-h exposure to environmental concentrations of carbaryl and fenitrothion, well below their NOAECs. A similar increase in the heart rate has been previously reported in fish embryos exposed to concentrations below the NOAEC of different pollutants (Agathokleous, 2022). For instance, an increase in heart beat has been found in marine medaka (*Oryzias melastigma*), Javanese medaka (*Oryzias javanicus*), and zebrafish embryos exposed to concentrations below the NOAEC for polystyrene microplastics, diuron and antibiotics, respectively (Chen et al., 2020a; Han et al., 2021; Ibrahim et al., 2020; Zhang et al., 2020). This stimulating effect on the heart rate, commonly no >60 % of the control, has been considered as the hormetic response of organisms to low dose of chemical stress (Agathokleous, 2022). Although the heart rate in zebrafish larvae and *D. magna* exhibits a biphasic response to fenitrothion, additional information would be needed to assess whether the increase in heart rate observed in these species after carbaryl exposure also follows a non-monotonic concentration-response (NMCR).

Modulation of the nuclear factor erythroid-2-related factor 2 (Nrf2) has been suggested to be the key mechanism behind the observed stimulation on heart rate (Agathokleous, 2022), although the precise mechanisms linking Nrf2 modulation and increased heart rate are still unknown. The teleost heart rate is controlled by the balance between excitatory effect of adrenergic fibers and the inhibitory effect of cholinergic fibers (Sandblom and Axelsson, 2011). The antagonism of carbaryl on α2B adrenoceptor

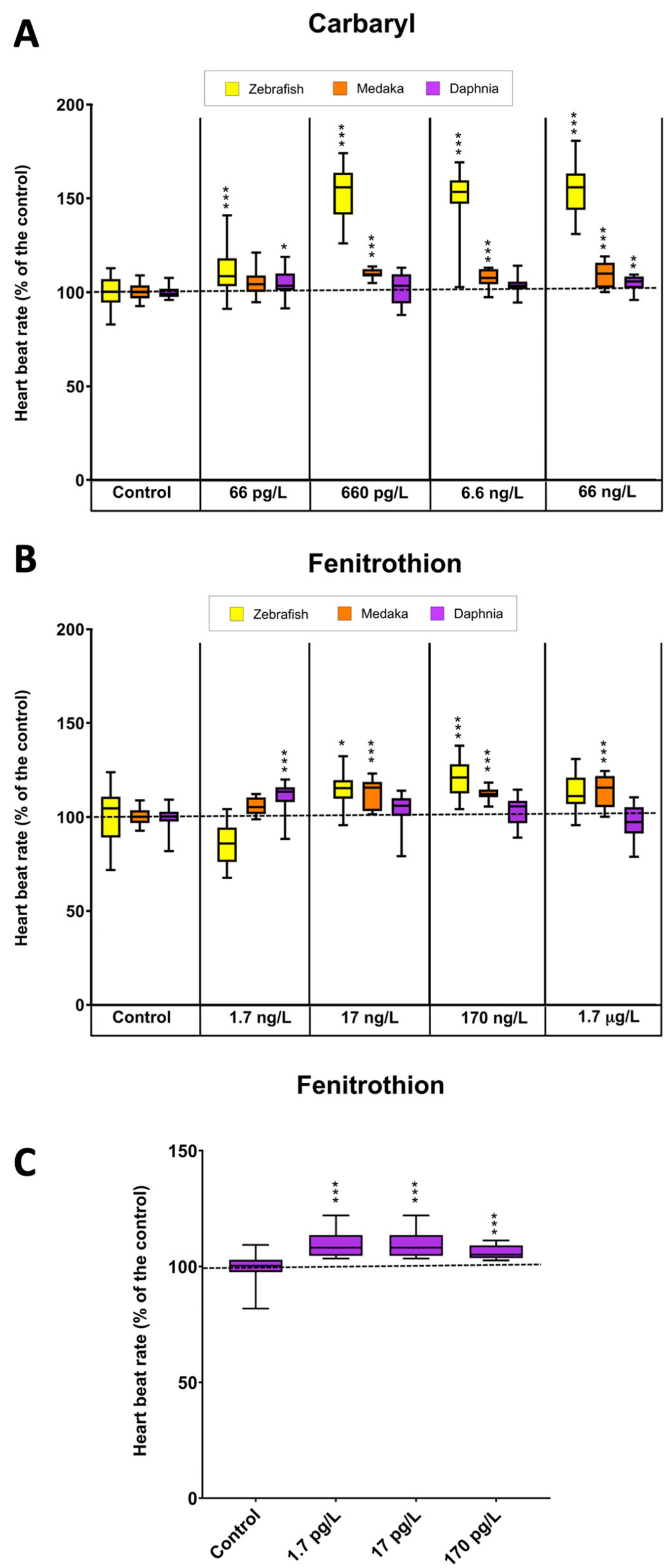

Fig. 1. Effect of 24 h exposure to environmental concentrations of carbaryl (A) and fenitrothion (B–C) on the heart rate in zebrafish larvae, Japanese medaka larvae and *D. magna*. A. Effect of carbaryl (66 pg/L-66 ng/L) on cardiac activity in the three selected species; B. Effect of fenitrothion (1.7 ng/L–1.7 μg/L) in the three selected species. C. Effect of fenitrothion (1.7–170 pg/L) on cardiac activity in *D. magna*; Boxplot representation, with the box indicating the 25th and 75th percentiles, the whiskers indicating the maximum and minimum values, and the thin line within the box indicating the median (*zebrafish*: n = 15–19 for carbaryl and n = 15–24 for fenitrothion; *Japanese medaka*: n = 7–11 for carbaryl and *n* = 11–14 for fenitrothion; *D. magna*: n = 15–38 for carbaryl and n = 11–49 for fenitrothion). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; Kruskal Wallis test with Bonferroni correction; Data from 2 to 3 independent experiments.

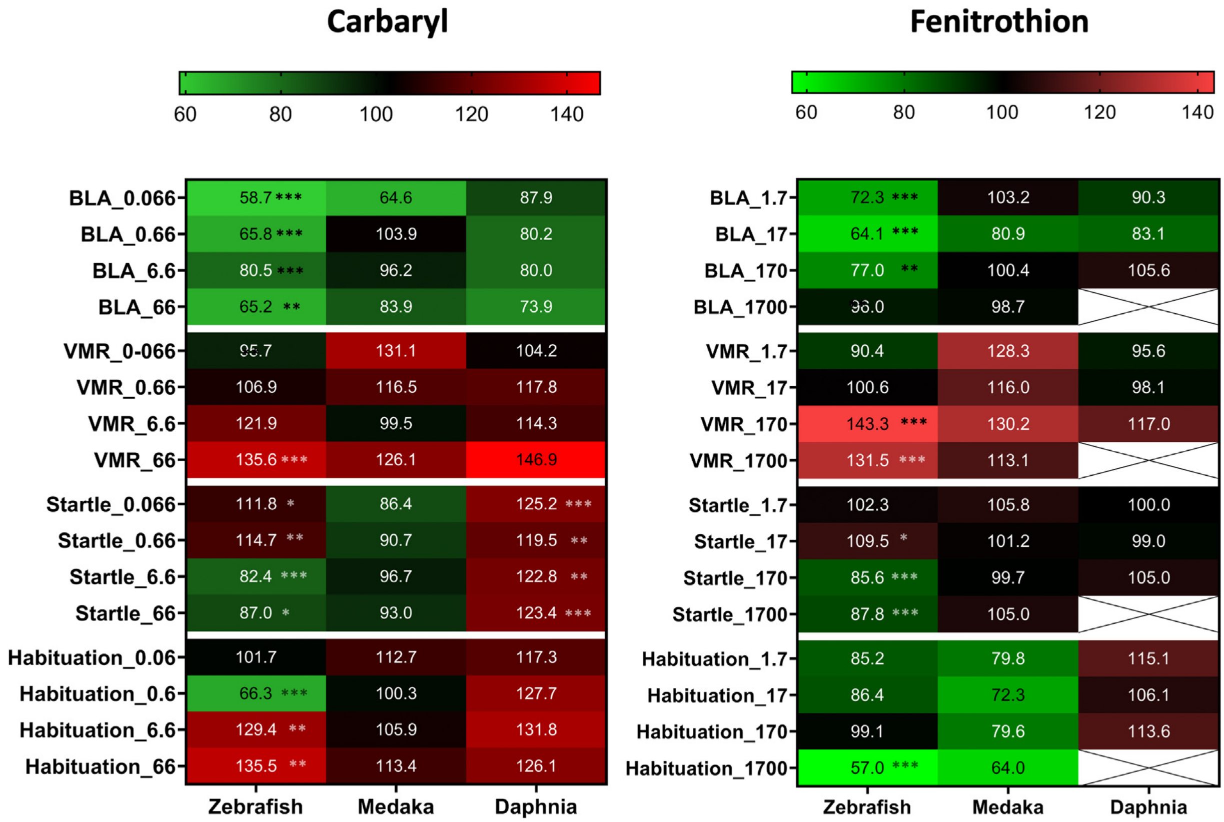

Fig. 2. Heat map diagram showing the changes in basal locomotor activity (BLA), visual motor response (VMR), startle response (Startle) and its habituation in zebrafish larvae, Japanese medaka larvae and *D. magna* after 24 h treatment with carbaryl (0.06–66 ng/L) and fenitrothion (1.7–1700 ng/L). Colors in the heat map represent the deviation from the control larvae (black color), with a gradient of greens or reds for values below or above the controls, respectively. The number inside each cell corresponds to the average of the results for each endpoint normalized as percentage of their respective controls [*zebrafish*: (1) Carbaryl: n = 83–158 for BLA, n = 79–153 for VMR, n = 61–136 for SR, and n = 40–73 for habituation; (2) Fenitrothion: n = 81–167 for BLA, n = 76–154 for VMR, n = 73–148 for SR, and n = 30–71 for habituation/*Japanese medaka*: (1) Carbaryl: n = 55–80 for BLA, n = 52–70 for VMR, n = 49–72 for SR, and n = 50–72 for habituation; (2) Fenitrothion: n = 28–47 for BLA, n = 30–49 for VMR, n = 37–51 for SR, and n = 37–49 for habituation/*D. magna*: (1) Carbaryl: n = 14–18 for BLA, n = 11–21 for VMR, n = 11–22 for SR, and n = 12–22 for habituation; (2) Fenitrothion: n = 16–18 for BLA, VMR, SR, and habituation]. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Kruskal Wallis test with Bonferroni correction; Data from 2 to 3 independent experiments.

(ADRA2B) and the serotonin 2B receptor (HTR2B) effect has recently been proposed as the molecular initiating event of the effect of this chemical on zebrafish heart rate (Faria et al., 2022). Although the results point to the fact that a similar mechanism could be behind the increased heart rate observed in Japanese medaka and *D. magna*, additional studies addressing this biological question are urgently needed. Moreover, a reduction in BLA and in the SR evoked by a vibrational stimulus has been reported in zebrafish larvae exposed to yohimbine and SB204741, prototypic antagonists of ADRA2B and HTR2B respectively (Faria et al., 2022), analogous to that produced by carbaryl and fenitrothion in this study. However, the fact that these drugs failed in producing any effect on VMR suggests that perhaps antagonism of these receptors is not the only mechanisms downstream of Nrf2 activation involved in the observed effects, and therefore additional pathways maybe modulating VMR. Although the binding of fenitrothion to the androgen receptor was suggested as a potential molecular initiating event of the behavioral effects on zebrafish larvae, more detailed studies discarded this hypothesis (Faria et al., 2021, 2022).

There is currently a consensus on the need to increase our understanding on how environmental concentrations of some pollutants can cause eco-neurotoxicity and how this differs between species (Ford et al., 2021; Legradi et al., 2018). Our results show that 24 h exposure to environmental concentrations of carbaryl and fenitrothion induced a significant hypolocomotion in zebrafish, and a similar trend in the other two species. In contrast, exposure to environmental concentrations of microplastics (MPs) resulted in hyperactivity in both adult zebrafish (Chen et al., 2020b) and the invertebrate *Brachionus plicatilis* (Gambardella et al., 2017). Moreover, exposure to relatively low concentrations of glyphosate also resulted in hyperactivity in rainbow trout larvae (*Oncorhynchus mykiss*) (Weeks Santos et al., 2019). Whereas the increase in locomotor activity has been suggested to be a general physiological response to low-level chemical stress (Agathokleous, 2022), the results presented in this manuscript do not support this hypothesis.

One interesting result from this work is that there is an uncoupling between heart rate and motor activity for all three model organisms, with an increase in heart rate despite to the observed trend to hypolocomotion. This result can be explained by the fact that, in contrast to adult fish in which cardiac activity is mainly determined by tissue metabolic demand, in fish embryos and early larvae, cardiac activity is still uncoupled from metabolic demand (Schwerte, 2009). While the modulation of cardiac activity by the metabolic demand in *D. magna* is not fully understood, different studies have shown increased heart rate and decreased locomotor activity in *Daphnia* exposed to low concentrations of chemicals (Bownik et al., 2017, 2020).

D. magna exposed for 24 h to 1.7 μg/L fenitrothion exhibited a severe phenotype, including a decrease in AChE activity of about 80 %. The fact that no effect on heart rate was observed in these animals suggests that heart rate is not under the control of the cholinergic system in this species. A decrease in heart rate was reported, however, in *Daphnia* 1 h after waterborne administration of acetylcholine, the AChE inhibitor tetraethylpyrophosphate and the muscarinic AChR agonist pilocarpine (Bekker and Krijgsman, 1951). Differences in the experimental design (exposure time, concentration of chemicals) might explain the observed differences. Additional studies should be performed to clarify the role of the cholinergic system in the heart rate control in *Daphnia*.

It is important to consider the differences in the startle assay used in this study for fish larvae and *D. magna* (see Supplementary Figs. S3–S5). While in fish the real startle response is that evoked by one acoustic/vibrational stimulus, *D. magna* has not a clear response to this type of stimulus. However, a sudden increase in light intensity is a strong aversive stimulus for *Daphnia*, and it is possible to evoke in this organism a startle-like escape response with a light flash. In fact, a test for assessing the effect of pollutants on the startle response evoked by a flash of light and its habituation in *D. magna* has recently been developed (Bedrossiantz et al., 2020). Therefore, the similar increase in the motor activity found in VMR and startle assays in *D. magna* can be explained by the use of the same aversive stimulus in both assays, light. In fact, the profile of the escape response evoked by light in the VMR was very similar between fish larvae and *D. magna*, emphasizing the conservation of the response to sudden changes in light intensity through evolution.

The ecological relevance of the observed effects of environmental concentrations of carbaryl and fenitrothion, well below their NOAECs, on the heart rate is quite important since effects occurred across phylogenetically very different species. In addition, the effects of these compounds on the selected behaviors may result in deleterious effects at short- and medium-term. First of all, as there is positive relationship between BLA and foraging efficiency (Mora-Zamorano et al., 2016), the hypolocomotion observed in the exposed organisms might result in a decrease in the foraging efficiency. VMR has been related with a response allowing organisms to avoid looming predators (Easter and Nicola, 1996) and so, the increase in VMR observed in the three organisms following exposure to concentrations of the two insecticides well below their NOAECs might attract the attention of nearby predators. Moreover, a significant relationship between the vibrational startle response and fish larval survival to predator strikes has been recently demonstrated (Fero et al., 2011), and in this regard the altered SR observed in the exposed fish larvae strongly suggests a decrease in the likelihood of surviving ambush predator strikes. Finally, habituation is a non-associative learning process by which organisms "learn" to ignore irrelevant stimuli commonly found in natural conditions (Best et al., 2008). Therefore, the increase in the habituation time found for some conditions in this study might result in a high energetic cost, whereas the decrease in the habituation time might result in the erroneous identification of a series of predator strikes as irrelevant stimuli, due to a too rapid habituation. Therefore, the results presented in this manuscript support the fact the outcome of the hormetic effects is not always beneficial, but also can be detrimental for the organisms, as previously proposed in different reviews on this phenomenon (Calabrese, 2010, 2011b).

5. Conclusions

In this study, changes in heart rate and selected behaviors have been found in three aquatic organisms, zebrafish, Japanese medaka and *D. magna*, exposed for only 24-h to concentrations of carbaryl and fenitrothion well below their NOAECs. Although carbaryl exhibited the highest potency, exposure to both pesticides led to strikingly similar effects on HR, BLA and VMR. Interestingly, even though the three selected species are phylogenetically very distant, the observed effects of these chemicals on heart rate, BLA and VMR were found to be very similar, suggesting the involvement of mechanisms well conserved throughout evolution. Therefore, results from this study emphasize the need of including the analysis of adverse effects in phylogenetically distant species in order to improve the predictivity of the results to other aquatic organisms. Finally, the potential adverse outcomes found in this work after the short-term exposure to environmental concentrations of carbaryl and fenitrothion, well below their NOAECs, emphasize the need to revise the NOAEC values for certain pollutants by integrating additional toxicological endpoints, such as behavioral responses or cardiac activity, into the predictive risk assessment methodology.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.161268.

Ethics statement

The animal study was reviewed and 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 11336). For the experiments with Japanese medaka at the University of Bordeaux, the authorization number was APAFIS#28934.

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CRediT authorship contribution statement

Juliette Bedrossiantz: Investigation, Formal analysis, Visualization, Writing – original draft. **Melissa Faria:** Investigation. **Eva Prats:** Investigation. **Carlos Barata:** Conceptualization, Writing – review & editing, Supervision. **Jérôme Cachot:** Conceptualization, Writing – review & editing, Supervision. **Demetrio Raldúa:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing, Supervision.

Data availability

Data will be made available on request.

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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