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Do you smell the danger? Effects of three commonly used pesticides on the olfactory-mediated antipredator response of zebrafish (*Danio rerio*)

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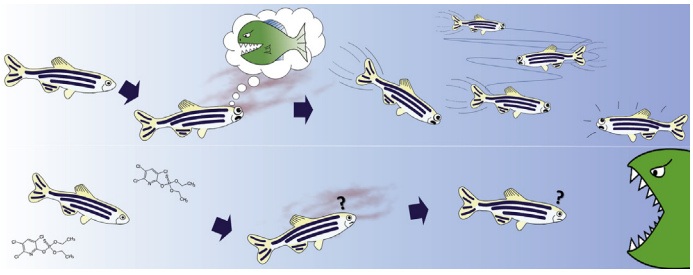
HIGHLIGHTS

- Pesticides alter the olfactory-mediated antipredator response of zebrafish in distinct manners.
- Chlorpyrifos impairs the antipredator behavior of zebrafish.
- Linuron alters the response to the conspecific skin extract.
- Zebrafish avoid permethrin in a choice maze.

GRAPHICAL ABSTRACT



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ABSTRACT

Fish are warned about the presence of predators via an alarm cue released from the skin of injured conspecifics. The detection of this odor inherently initiates an antipredator response, which increases the chance of survival for the individual. In the present study, we assessed the effect of three commonly used pesticides on the antipredator response of zebrafish (*Danio rerio*). For this, we analyzed the behavioral response of zebrafish to a conspecific skin extract following 24 h of exposure to the respective contaminants. Results demonstrate that fish exposed to 20 μg/L of the organophosphate insecticide chlorpyrifos significantly reduced bottom-dwelling and freezing behavior, suggesting an impairment of the antipredator response. For the urea-herbicide linuron and the pyrethroid insecticide permethrin, no statistically significant effects could be detected. However, linuron-exposed fish appeared to respond in an altered manner to the skin extract; some individuals failed to perform the inherent behaviors such as erratic movements and instead merely increased their velocity. Furthermore, we determined whether zebrafish would avoid the pesticides in a choice maze. While fish avoided permethrin, they behaved indifferently to chlorpyrifos and linuron. The study demonstrates that pesticides may alter the olfactory-

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mediated antipredator response of zebrafish in distinct ways, revealing that particularly fish exposed to chlorpyrifos may be more prone to predation.

1. Introduction

Sensory perception enables organisms to connect with their environment and gather information that is essential to every aspect of their lives. While humans heavily rely on their visual and auditory senses (San Roque et al., 2015), other life forms, such as fish, experience their surroundings largely via the sense of smell (Hara, 1975; Ache and Young, 2005; Ashwell, 2012; Nielsen et al., 2015; Silva et al., 2016).

The olfactory system of most teleost fish is able to detect a wide variety of dissolved molecules and distinguish between different odors very precisely (Hara, 1975; Bargmann, 2006). Thereby, chemosensation is often closely linked to behavior. In fact, it has been shown that certain odors initiate an innate behavioral response in many organisms. For instance, predator odors trigger fright responses in naive European rabbits (*Oryctolagus cuniculus*) (Monclús et al., 2005) and highly domesticated hatchery-raised rainbow trout (*Oncorhynchus mykiss*) (Kopack et al., 2015), which had never experienced predation.

Escaping predation is essential for the survival of any organism. Fish belonging to the superorder Ostariophysi are warned about the presence of predators via a so-called *Schreckstoff* or alarm substance released out of epidermal club cells in the skin of an injured conspecific (Frisch, 1938; Smith, 1992). For a number of teleost species including fathead minnow (*Pimephales promelas*) (Chivers and Smith, 1993) and crucian carp (*Carassius carassius*) (Hamdani et al., 2000) it was demonstrated that this cue is perceived via the olfactory system (Porteus et al., 2018; Williams et al., 2019) and initiates an antipredator response that is specific for the respective fish species (Smith, 1992). If an individual fails to properly respond to the alarm substance, chances of survival will decrease substantially (Mathis and Smith, 1993; Dill, 1998; McIntyre et al., 2012).

In the last decades, numerous studies investigated the effect of metals on olfaction and related behaviors (Hansen et al., 1999; Scott et al., 2003; McPherson et al., 2004; Carreau and Pyle, 2005; Mirza et al., 2009; Wang et al., 2013; Williams and Gallagher, 2013; Dew et al., 2014, 2016), demonstrating a metal-induced olfactory impairment at environmentally relevant concentrations. Less work has been published addressing the effect of pesticides on the olfactory system; nonetheless, it shows similar findings. For instance, following exposure to the carbamate fungicide IPBC (iodopropynyl butylcarbamate), coho salmon (*Oncorhynchus kisutch*) failed to respond to an alarm cue, which may increase their susceptibility to predation (Tierney et al., 2006).

For chlorpyrifos, an organophosphate insecticide used in many countries worldwide, effects on neuronal development (Zhang et al., 2015; Abdelmalek et al., 2016) and behavior (Sledge et al., 2011; Qiu et al., 2017) in fish and rodents are well described. Furthermore, chlorpyrifos altered the expression of several genes in the olfactory system of zebrafish (*Danio rerio*) (Tilton et al., 2011b). In addition to its anti-androgenic effects (Lambright et al., 2000; Marlatt et al., 2013), the urea-herbicide linuron was shown to impair the electrophysiological response of three different salmonids to the social cue taurocholic acid (TCA) but not to the amino acid L-serine (Tierney et al., 2007a); the latter washes off the skin from mammals and is usually avoided by salmonids (Idler et al., 1956; Rehnberg and Schreck, 1987; Tierney et al., 2007a). Being a pyrethroid pesticide, permethrin impedes the closing of sodium channels in the nervous system (Davies et al., 2007), thereby causing a continuous excitation that can ultimately lead to paralysis and death (Field et al., 2017). Cypermethrin, a structurally similar pyrethroid insecticide, disrupted the endocrine response of male brown trout (*Salmo trutta* L.) to female reproductive pheromones (Jaensson et al., 2007).

In a number of studies, the effect of pesticides on the antipredator response of fish was assessed by studying the avoidance of a conspecific skin extract. Yet, little is known about how pesticides affect the different behaviors typically associated with the antipredator response, such as bottom-dwelling and freezing. In the last years, zebrafish, a well-studied model organism widely used in neurobiology, ecotoxicology, pharmacology, and medicine, has increasingly been used in behavioral studies (Kalueff et al., 2013; Legradi et al., 2018). Especially its antipredator response has been investigated in the context of anxiety and anxiolytic drugs (Gerlai, 2010, 2013) and efforts were made to standardize the analysis (Cachat et al., 2010; Kalueff et al., 2013). Since its olfactory system is typical for many teleost fish (Hansen and Zeiske, 1993; Baier and Korsching, 1994), zebrafish presents a very suitable model for investigating the effects of pesticides on the olfactory-mediated alarm response. Beyond that, its small size favors parallelized testing, making behavioral experiments less labor and time-consuming.

The aim of the present study was to assess the antipredator response of zebrafish following exposure to three commonly used pesticides, namely linuron, chlorpyrifos, and permethrin. In doing so, this work supports the establishment of zebrafish as a model for the assessment of pesticide-induced effects on olfactory-mediated behaviors. We exposed adult zebrafish for 24 h to different concentrations of the contaminants and recorded their behavior prior and following the delivery of a conspecific skin extract. Performing both automated and manual analysis, we measured different elements of the antipredator response – bottom-dwelling, erratic movements, and freezing – in order to get a holistic picture of the effect of the pesticides on this complex behavior. In order to automate and accelerate the analysis of the behavioral data, we developed routines in R. Furthermore, we performed choice maze experiments to answer the question of whether the fish would choose to avoid exposure to the pesticides if possible.

2. Experimental procedures

2.1. Animals

Adult zebrafish were kept on a light-dark cycle of 14:10 h in the aquatic facility at RWTH Aachen University according to the method described by Braunbeck et al. (2005). The holding water was generated from tap water via reverse osmosis and the addition of a salt mixture (HS aqua Marin Pro Salt). Water was changed once a week and the temperature was kept at 26 ± 1 °C. Animals were fed *ad libitum* twice a day with commercial TetraMin flakes (Tetra GmbH, Melle, Germany). All experiments were conducted in accordance with the Animal Welfare Act and with permission of the federal authorities (Ministry for Environment, Agriculture, Conservation and Consumer Protection of the State of North Rhine-

Westphalia, Germany, registration number 84–02.04.2016. A082). At the end of the experiment, zebrafish were euthanized according to procedures approved by animal welfare (TVT 2010) (Tierärztliche Vereinigung für Tierschutz e.V., 2010).

2.2. Chemicals and exposure

All chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and were of analytical grade. Stock solutions were prepared in dimethyl sulfoxide (DMSO) and test solutions and solvent controls had a final DMSO concentration of 0.01% (v/v). All experimental treatment solutions were prepared with reconstituted water (demineralized water supplemented with HS aqua Marin Pro Salt). Maximal exposure concentrations were chosen to be 10% of the 96 h-LC50 of each chemical, the lowest ones were selected to approach environmentally relevant concentrations: chlorpyrifos: 2, 5, and 20 μg/L and linuron: 10, 100, and 300 μg/L. Since for permethrin the highest test concentration of 250 ng/L did not result in any significant effects in the present or other studies conducted in our lab, we decided not to test lower concentrations in order to reduce the number of animals required. To minimize the loss of chemicals due to adsorption to the glass, test vessels were incubated with the respective treatment solutions 24 h prior to the experiment and solutions were exchanged immediately before the beginning of the exposure. Adult zebrafish were exposed for 24 h to the respective pesticide. Treatment solutions were sampled at the beginning (except for permethrin) and end of each exposure period and the pesticide concentrations were analyzed by gas chromatography–mass spectrometry (GC-MS; chlorpyrifos, permethrin) or liquid chromatography–mass spectrometry (LC-MS; linuron) to verify nominal concentrations (for a detailed description see supplementary material). For the ease of reading, nominal concentrations are used in the following text and figures.

2.3. Skin extract preparation

Skin extract was prepared from dead control fish used in previous experiments that were washed in water, snap-frozen in liquid nitrogen, and stored at −80 °C. Fish were thawed on ice and the skin was removed using surgical scissors and forceps. Extra care was taken to prevent the addition of other tissue or blood to the skin sample. For 60 mL of skin extract the skin of four fish (two males and two females) was used (approximately 12 cm^2), demineralized water was added and the skin was homogenized (homogenizer VDI 12, S12N-5S, VWR International GmbH, Darmstadt, Germany) and filtered through a glass microfiber filter. The skin extract was stored on ice until usage and prepared fresh on each experimental day.

2.4. Antipredator study

Antipredator experiments were conducted in parallel in two 35 L observation tanks, which were obscured with black silicon on three sides from the outside to prevent any visual disturbance. Additionally, the tanks were placed in a shelf covered from all but one side with cardboard and an opaque curtain on the front. Tubing was attached, facilitating the addition of stimuli to the observation tanks from behind the curtain. A camera (Rollei Actioncams 426, 4k, Rollei GmbH & Co. KG, Norderstedt, Germany) was installed in front of each observation tank to record the fish during the whole experiment. The observation tanks were filled with reconstituted water. Following 24 h of exposure, zebrafish were introduced individually into the tanks and left undisturbed for an acclimation period of 40 min prior to stimulus delivery. Mathuru et al. (2012) demonstrated that despite zebrafish being a shoaling fish, the behavior of individuals was either similar to that of schools or more drastic with individual fish showing increased anxiety and/or alternative strategies. Thus, by measuring individual zebrafish we could reduce the number of animals required while getting similar or more pronounced responses to conspecific skin extract. 7 min prior to the delivery of the stimulus solution, the recording was started. As a blank stimulus, reconstituted water was used to assess whether the addition of a liquid alone triggers an alarm response via visual or mechanical stimulation due to the resulting movement of the water and formation of air bubbles. The conspecific skin extract was used to induce the antipredator response. 5 mL of either stimulus was delivered to the observation tanks via the tubing and the tubing was flushed with additional 5 mL of water. Subsequently, fish behavior was recorded for 7 min. Between trials, tanks were emptied, flushed with 5 L of water, and filled with 30 L of fresh reconstituted water prior to the introduction of the next fish. 20 animals were used for each treatment.

2.5. Behavioral analysis

The recorded behavior was analyzed using EthoVision® XT 11.5 (Noldus Information Technology bv, Wageningen, The Netherlands) and R version 3.5.1 (R Developmental Core Team, 2018). Fish behavior was tracked 7 min prior and after stimulus delivery (according to Parra et al., 2009), hereinafter referred to as pre-stimulus and post-stimulus. To assess bottom-dwelling, the observation tanks were divided into an upper and lower half and time spent in the lower half was recorded. Raw data were exported as excel files and statistically analyzed using R. The behaviors investigated were: time spent in the bottom half of the observation tank, time spent freezing, and duration of erratic movements (fast swimming bursts with multiple changes of direction).

As manual observation is very time-consuming and prone to subjective interpretations, we developed routines in R to automate the analysis of bottom-dwelling and freezing. Thereby, freezing was defined as a time period of at least 5 s in which the velocity did not exceed 2 cm/s and 90% of all velocity values were below 0.5 cm/s. As erratic movements could not be identified automatically, they were manually recorded from the video. For both, freezing and erratic movements, post-stimulus values of each fish were corrected by subtracting the respective pre-stimulus values. In order to assess all behavioral elements of the antipredator response as a whole, we created a scoring system which included all of the different behaviors shown by the individual fish as well as the time spent displaying these behaviors. As bottom-dwelling appeared to be least specific for the antipredator response, it was assigned the lowest score value (>80% of time post-stimulus = 1), followed by erratic movements (>5 s = 4, >30 s = 6, >180 s = 8), and freezing (>5 s = 6, >30 s = 8, >180 s = 10), often being the last stage of the response. Finally, the sum of the score values was calculated for each fish.

2.6. Choice maze experiment

We used a custom-made y maze (for further details please refer to the supplementary material) to assess whether zebrafish would avoid the selected pesticides when given a choice. Briefly, an individual zebrafish was transferred into a separated acclimation zone at the lower end of the start-arm and left undisturbed for 20 min. Subsequently, 80 mL of the contaminant solution plus 20 mL of water were delivered to one of the two cue-receiving arms and 100 mL of water to the other one (blank). The contaminant-receiving arm was randomly chosen. As contaminant stimuli, either 20 μg/L chlorpyrifos, 300 μg/L linuron or 250 ng/L

permethrin were used. Then, the gate separating the acclimation zone from the rest of the maze was lifted and the fish was recorded on video for 10 min. After the trial, fish were maintained in tanks until further usage in a different experiment. 28–29 fish were tested per contaminant stimulus.

2.7. Statistical analysis

The statistical analysis of the individual behaviors as well as the scoring system was performed using R Version 3.5.1 and graphs were prepared using the ggplot2 package (Wickham, 2016) and cowplot package (Wilke, 2019). To validate the suitability of the behavioral endpoints for the assessment of the antipredator response to the conspecific skin extract, pre-stimulus data of the skin extract and water solvent control were tested against the post-stimulus data for each endpoint using Wilcoxon tests. Since neither the behavioral data nor the results of the scoring system conformed to parametric assumptions, Kruskal-Wallis tests followed by one-sided Wilcoxon tests were used. Since the skin extract solvent control showed the normal response of unexposed animals to the stimulus, it functioned as a negative control in the present study. Thus, statistical comparisons were always made between the skin extract solvent control and the treatments. In some cases, Dunn's test (dunn.test package) (Dinno, 2017) was used to conduct pair-wise comparisons. The Benjamini-Hochberg p-values adjustment was used to correct for multiple testing where necessary. For the multivariate analysis of the antipredator response, a Multiple Response Permutation Procedure (MRPP) (Mielke et al., 1976) was conducted with PC-ORD (MjM Software, Gleneden Beach, Oregon) using Euclidean distance as the distance measure. For the choice maze experiment, paired t-tests were carried out to compare the time spent in the contaminant versus the blank arm.

3. Results and discussion

3.1. Behavioral elements of the antipredator response

Nominal and measured concentrations of the pesticides were in reasonable agreement at the beginning of the exposure (please refer to Table 1 in supplementary material). Solvent control fish displayed no significant change in any of the behaviors investigated after the delivery of water to the observation tanks. In contrast, following the addition of skin extract, zebrafish increased bottom-dwelling ($p = 0.00004$), the percentage of time spent with erratic movements ($p = 0.00008$), and the percentage of time spent freezing ($p = 0.0002$). Thus, bottom-dwelling, erratic movements, and freezing are suitable endpoints for the assessment of the olfactory-mediated antipredator response. Following exposure to the pesticides, no statistically significant differences in bottom-dwelling, erratic movements, and freezing were detected between the solvent controls and the treatments prior to the stimulus addition. Bottom-dwelling proved to be a robust indicator of fright- and anxiety-like behaviors in fish and is described as part of the antipredator response for many fish species including zebrafish (Schutz, 1956; Smith, 1992; Berejikian et al., 1999; Speedie and Gerlai, 2008; Canzian et al., 2017); the present study is consistent with these findings. Following the delivery of conspecific skin extract, zebrafish exposed to 0.01% DMSO (solvent control) spent significantly more time in the bottom half of the tank, compared to those stimulated with water ($p = 0.001$) (Fig. 1).

While zebrafish exposed to 100 μg/L linuron showed a decrease in time spent in the lower half of the tank compared to after exposure to 10 μg/L ($p = 0.046$), the time spent bottom-dwelling at 300 μg/L increased back to the level of the skin extract solvent control. Following exposure to chlorpyrifos, the time spent in the bottom half of the tank declined in a concentration-dependent manner to a median of 67% ($p = 0.02$). Staying closer to the bottom might help the fish to blend in with the ground, or in combination with erratic movements, to stir up sediment and debris, enabling the fish to hide in the turbid water (Speedie and Gerlai, 2008). A failure to perform this behavior could make the individual fish more susceptible to predation. Individuals swimming in higher areas of the water column would be clearly visible for predators hunting from above, such as piscivorous birds. For permethrin, no significant effect on bottom-dwelling could be detected at 250 ng/L, which corresponds to 10% of the 96 h-LC50 of this insecticide.

Control fish stimulated with skin extract spent more time performing both erratic movements and freezing than fish stimulated with water ($p = 0.003$ and $p = 0.013$, respectively; Fig. 2). Following exposure to linuron, no significant changes in the duration of erratic movements or time spent freezing could be detected. After treatment with 20 μg/L chlorpyrifos, zebrafish spent less time freezing ($p = 0.013$). For permethrin, neither the time spent

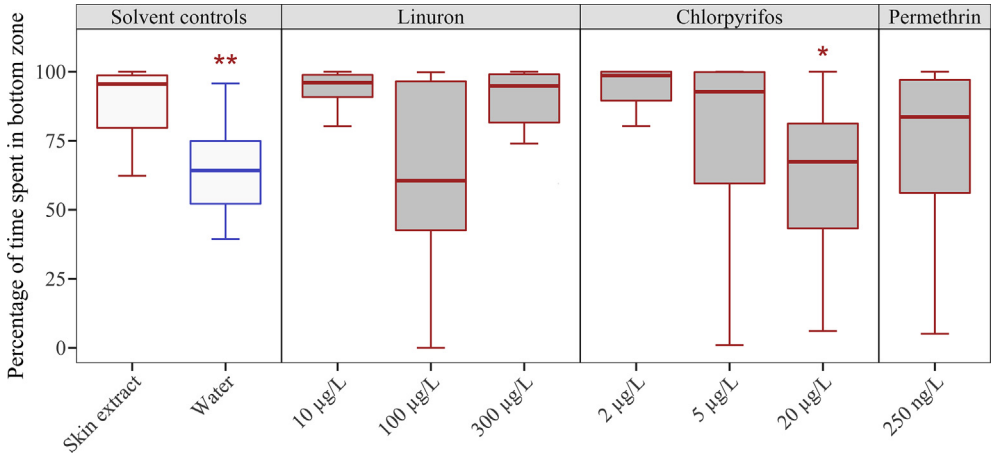

Fig. 1. Percentage of time zebrafish spent in the bottom half of the tank following 24 h of exposure to pesticides and delivery of conspecific skin extract (red lines) as an alarm cue. In the case of the water solvent control, water (blue lines) was delivered as a blank stimulus. Statistical differences between the skin extract solvent control (white box, red lines) and the water solvent control (white box, blue lines) as well as the pesticide treatments (gray boxes) are denoted with asterisks. *$p < 0.05$, **$p < 0.01$. $n = 20$.

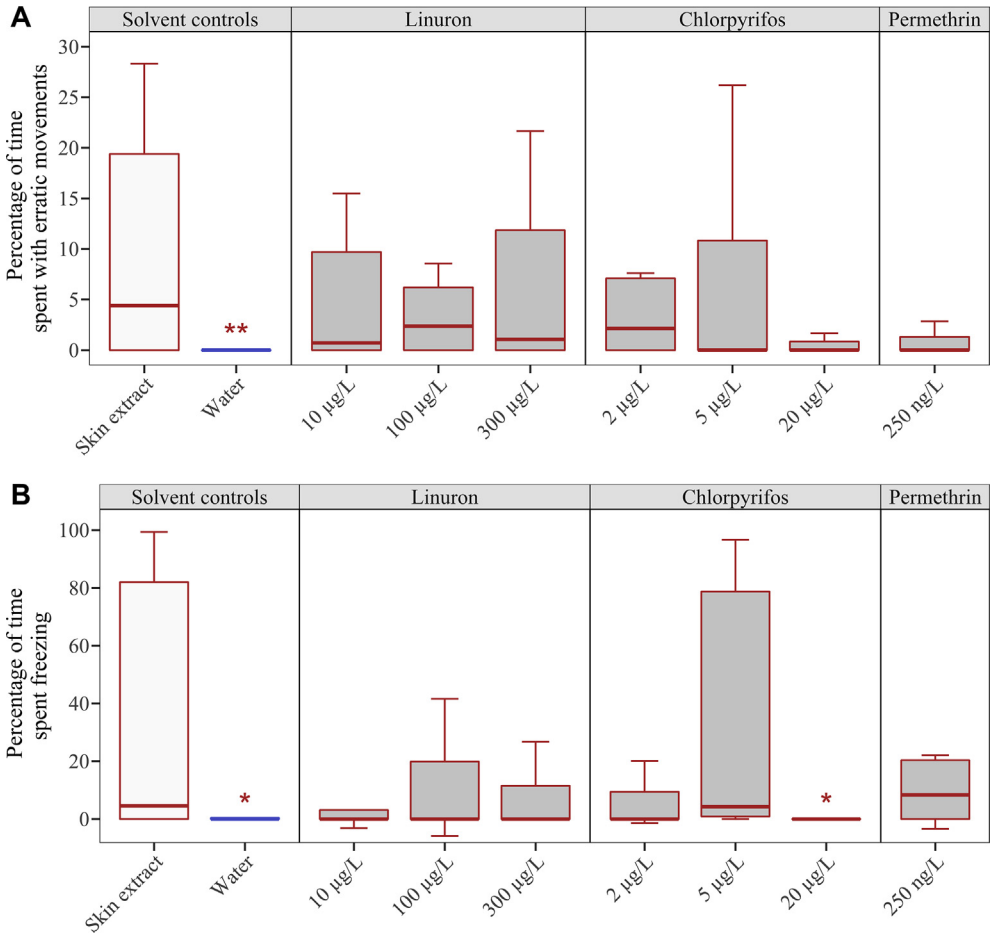

Fig. 2. Percentage of time zebrafish spent displaying erratic movements (**A**) or freezing (**B**) following 24 h of exposure to pesticides and delivery of conspecific skin extract (red lines) as an alarm cue. In the case of the water solvent control, water (blue lines) was delivered as a blank stimulus. Statistical differences between the skin extract solvent control (white box, red lines) and the water solvent control (white box, blue lines) as well as the pesticide treatments (gray boxes) are denoted with asterisks. *$p < 0.05$, **$p < 0.01$. $n = 20$.

freezing nor performing erratic movements was different from the skin extract solvent control.

The multivariate analysis (MRPP with Euclidean distance) showed a highly significant difference ($p = 0.000004$). Pairwise comparisons were performed to detect statistically significant distinctions between the treatments (for all of the results see supplementary material). Comparing the skin extract solvent control with the water solvent control as well as the pesticide treatments, statistically significant differences were detected for the water solvent control ($p = 0.000003$), 20 µg/L chlorpyrifos ($p = 0.002$), and 100 µg/L linuron ($p = 0.047$).

3.2. Scoring system

In addition, we created a scoring system combining all of the three behaviors measured; score values were assigned according to the severity of the behavior (bottom-dwelling < erratic movements < freezing) as well as the time spent displaying it. In doing so, differences in the antipredator behavior of zebrafish became more distinct (Fig. 3). After the delivery of skin extract, score values of the solvent control were significantly higher than following the addition of water ($p = 0.0003$). Of all pesticide treatments, only 20 µg/L chlorpyrifos proved to be significantly different from the skin extract solvent control ($p = 0.0009$).

The reduction in both bottom-dwelling and duration of freezing as a response to the conspecific alarm cue following exposure to 20 µg/L chlorpyrifos, as well as the low score values, suggest an impairment of the antipredator response. Chlorpyrifos is known to reduce the electroolfactography (EOG) response of juvenile rainbow trout to the conspecific cue TCA and the avoidance of the amino acid L-serine (Maryoung et al., 2015). By assessing paired electrophysiological recordings from the olfactory epithelium (OE) and olfactory bulb of juvenile coho salmon following a 7-day exposure to 2.5 µg/L chlorpyrifos, it was demonstrated that the pesticide directly affects signal transduction within the olfactory sensory neurons (OSNs) (Sandahl et al., 2004). The present study is in line with these findings, as the fact that fish did not respond to the conspecific skin extract following exposure to 20 µg/L chlorpyrifos may indicate a failure to perceive the latter. The general mode of action of chlorpyrifos is the inhibition of acetylcholinesterase (AChE), which is known to be expressed in the olfactory system of chinook salmon (*Oncorhynchus tshawytscha*) (Sandahl et al., 2004). Moreover, other AChE inhibitors such as diazinon and carbofuran also impair olfaction in Atlantic salmon (*Salmo salar*) (Moore and Waring, 1996; Waring and Moore, 1997). Likewise, diazinon was shown to significantly affect the antipredator response of chinook salmon (Scholz et al., 2000). One proposed role of acetylcholine (ACh) in the OE is the modulation of neighboring supporting cells and OSNs by cholinergic, nonneuronal microvillous cells (Ogura et al., 2011). Using calcium imaging, it was shown

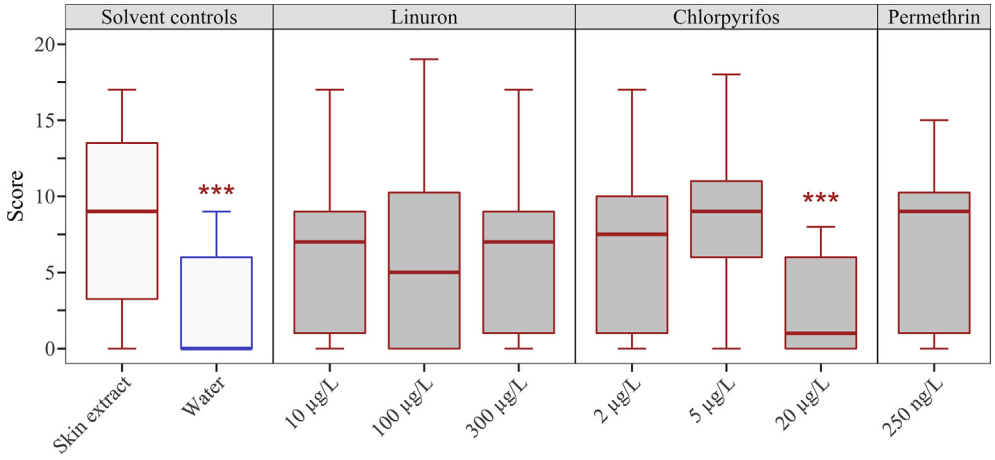

Fig. 3. Score values for the behavioral response of zebrafish following 24 h of exposure to pesticides and delivery of conspecific skin extract (red lines) as an alarm cue. In the case of the water solvent control, water (blue lines) was delivered as a blank stimulus. Bottom-dwelling was assigned the lowest score value (>80% of time post-stimulus = 1), followed by erratic movements (>5s = 4, >30 s = 6, >180 s = 8), and freezing (>5 s = 6, >30 s = 8, >180 s = 10). The sum of the score values was calculated for each fish. Statistical differences between the skin extract solvent control (white box, red lines) and the water solvent control (white box, blue lines) as well as the pesticide treatments (gray boxes) are denoted with asterisks. ***$p < 0.001$. n = 20. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

that intracellular calcium levels increased following induction by ACh in isolated supporting cells. In contrast, in some OSNs ACh inhibited the induction of calcium level increases by the adenylyl cyclase activator forskolin (Ogura et al., 2011). Therefore, an increased ACh level as a consequence of AChE inhibition by chlorpyrifos could inhibit the signal transduction subsequent to the binding of the antipredator cue to the olfactory receptors of ciliated OSNs. However, this hypothesis has to be thoroughly tested in further experiments. For instance, AChE activity in the OE should be measured to address whether it is reduced following exposure to chlorpyrifos. Apart from the effects on the olfactory system, the inhibition of AChE in the muscles and brain of zebrafish could also cause behavioral changes. Following 24 h of treatment with 35–220 µg/L chlorpyrifos, AChE activity in zebrafish muscles was shown to decrease to 29% of the control levels; however, swimming rates were only affected at 220 µg/L (Tilton et al., 2011a). Tierney et al. (2007b) demonstrated a significant reduction of brain AChE in coho salmon at exposures of 10 µg/L or greater for 96 h. For salmonids, the 96 h-LC50 of chlorpyrifos has been reported to range between 7.1 µg/L (Johnson and Finley, 1980) and 51 µg/L (Macek et al., 1969), whereas for zebrafish it was determined to be 289 µg/L (Singh et al., 2017). Taking the higher sensitivity of salmonids to chlorpyrifos as well as the longer exposure duration in the study of Tierney et al. (2007b) into account, we conclude that the inhibition of brain AChE did probably not play a role in the behavioral alterations measured in the present study.

Permethrin did not induce significant effects on any of the behaviors tested and thus, did not alter the antipredator response of zebrafish. This may be explained by the low exposure concentration used in the present study, which may have been below the threshold for sublethal toxicity. Permethrin is highly toxic for fish and the concentration used in this study corresponds to 10% of the 96 h-LC50 for zebrafish (Zhang et al., 2010). Thus, exposing zebrafish to higher concentrations of permethrin would likely cause mortality. To the best of our knowledge, no other studies to date address the effect of permethrin on the antipredator behavior or olfaction of fish. For esfenvalerate, another pyrethroid, no effect on the EOG responses to TCA or L-serine could be detected; however, some fish displayed irregular bursts of activity in the olfactory bulb (Sandahl et al., 2004). Following 5 days of exposure to <0.004 µg/L cypermethrin, mature male salmon parr displayed a reduction in the EOG response to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Moore and Waring, 2001). Consequently, there is evidence suggesting that pyrethroids alter olfaction in fish at very low concentrations. Up to now, it is not known for certain which OSNs are responsible for the detection of pheromones in fish; nonetheless, crypt neurons are thought to be conceivable candidates (Hamdani and Døving, 2006; Ahuja et al., 2013). Consequently, it is possible that pyrethroids differentially impair crypt OSNs. EOGs after exposure to permethrin using stimuli which are detected by distinct OSN classes could be performed in the future to reveal such an OSN specific effect.

3.3. Automated vs. manual data analysis

In the current study, we tried to automate as much of the data analysis as possible in order to reduce the immense amount of time required to manually evaluate the recordings. Our final goal was to write a script, which automatically extracts behaviors of interest from the raw data, analyzes them statistically, and summarizes the results in tables and plots. Using tracking software as EthoVision XT, a variety of variables can be measured very precisely. Some behaviors can be easily analyzed using one or a combination of these variables; for instance the variable “in zone” is very well suited to assess bottom-dwelling. Others, such as erratic movements, are considerably harder to define (Gerlai, 2013). Although some variables, such as turn angle, velocity, and the variability of both, are promising for describing this behavior, we did not find a computation of these variables that reliably detected erratic movements. Beyond that, the sole use of automated analysis can also be disadvantageous. In order to extract changes in behavior from the variety of variables measured, it is essential to know the behavior in advance and to be able to clearly define it with the variables. This could mean that unknown or poorly defined movement patterns might be overlooked. Additionally, given the high variability of behavioral data, subtle distinctions in the behavior might get lost in the data noise.

By manually analyzing the videos, we noticed a difference between fish treated with linuron and those exposed to permethrin or chlorpyrifos. While fish generally seemed to either react displaying the typical antipredator behavior or not at all, individuals treated with linuron almost always appeared to perceive the stimulus and respond to it; however, they partly failed to perform the inherent behavior. Instead, they appeared to be alarmed and increased their velocity ($p = 0.0005$ for 300 µg/L linuron compared to the water

solvent control; see Fig. 2 in supplementary material) but did not always show erratic movements and freezing. Thus, we hypothesized that the conspecific skin extract, or at least some odorants of that complex mixture, were able to bind to the odorant receptors and initiate the signal cascade, but in some cases, the interpretation in the olfactory bulb or higher brain regions was altered. Linuron was shown to differentially impair the EOG response to distinct odorants – while 100 μg/L linuron did not affect TCA-evoked responses, exposure to 10 μg/L resulted in a 50% inhibition of the response to L-serine (Tierney et al., 2007a). TCA and L-serine are known to be perceived by different classes of OSNs, namely ciliated and microvillous OSNs (Døving et al., 2011; Laframboise and Zielinski, 2011). While microvillous OSNs are specialized in the detection of amino acids, ciliated OSNs have a broader spectrum of ligands. For fathead minnows, it has been shown that the skin extract is perceived by ciliated OSNs (Dew et al., 2014). This is in line with our study, as linuron-exposed fish still reacted to the delivery of the stimulus. Nevertheless, further research is needed to address the question of why some fish exposed to linuron failed to respond in a typical manner. The combination of physiological measurements in the olfactory rosette and the olfactory bulb could reveal whether linuron affects the signal transduction downstream of the OE rather than the binding of odorants to the odorant receptor. A study assessing the effects of diuron, a structurally closely related herbicide, on goldfish demonstrated increased burst swimming following 24 h of exposure to 50 μg/L (Saglio et al., 1996). For zebrafish larvae, an increase in locomotion in the dark phase of the light:dark transition test was observed after treatment with 1 mg/L diuron (Velki et al., 2017), however, the underlying mechanisms of the altered swimming behavior are not yet known.

3.4. Choice maze

Choice maze experiments were performed to assess whether zebrafish would avoid the three pesticides if possible (Fig. 4). Avoidance responses can be mediated by gustation, solitary chemosensory cells, nociception at the gills, or olfaction; most scientists assume avoidance being mainly mediated via the latter (Tierney, 2016). When either chlorpyrifos or linuron was delivered to one arm of the y maze, the time zebrafish spent in the contaminant and blank arm did not differ. This could be due to two reasons. First, fish might not have been able to detect chlorpyrifos and linuron at the concentrations delivered. Second, fish might have perceived the pesticides, but not experienced them as either unpleasant or pleasant. Thus, they might not have avoided them or have been attracted by them. Studies using sheepshead minnows (*Cyprinodon variegatus*) (Hansen, 1969) and mosquitofish (*Gambusia affinis*) (Hansen et al., 1972) showed that these were indifferent to 10 μg/L chlorpyrifos but avoided higher concentrations (100 μg/L). We therefore assume that in the present study, the threshold concentration of chlorpyrifos triggering avoidance behavior in zebrafish was not yet reached. Following delivery of 250 ng/L permethrin, zebrafish spent significantly less time in the contaminant arm compared to the blank arm ($p = 0.031$). This was surprising for us, as the concentration of the stimulus was very low and avoidance responses below the μg/L range are rare. Nevertheless, rainbow trout were shown to avoid copper sulfate in concentrations as low as 100 ng/L (Folmar, 1976). The surfactant sodium lauryl sulfate induced avoidance behavior already at 10 ng/L (Ishida and Kobayashi, 1995). Future research should perform EOG experiments using permethrin as a stimulus to support the hypothesis that zebrafish can detect this pyrethroid in such low concentrations. In the environment, our results would mean that spills or other point sources of permethrin may be avoided by the fish and, thus, not affect them. In the case of chlorpyrifos and linuron, however, fish would be more likely to be exposed to those contaminants.

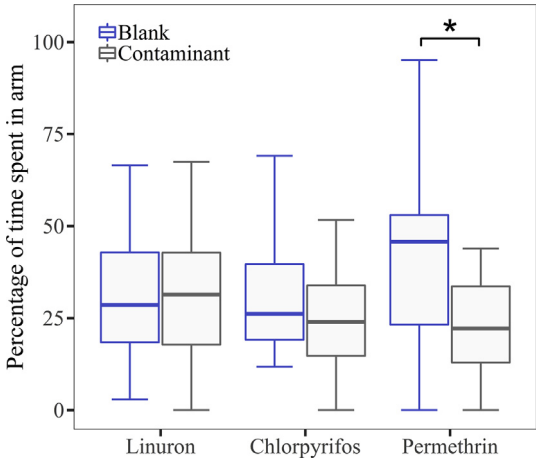

Fig. 4. Percentage of time zebrafish spent in blank (blue lines) and contaminant-receiving arm (gray lines) of a choice maze. As a stimulus either 80 mL of water or contaminant (20 μg/L chlorpyrifos, 300 μg/L linuron, and 250 ng/L permethrin) was used. Statistical differences are denoted with asterisks. *$p < 0.05$. Chlorpyrifos: $n = 28$, linuron and permethrin: $n = 29$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.5. Olfactory endpoints vs other toxicity endpoints

The olfactory system of fish is thought to be particularly vulnerable to pollution as the OSNs are just covered by a thin layer of mucous and, thus, in almost direct contact with the ambient water. To evaluate whether the olfactory-mediated antipredator response is especially sensitive to chlorpyrifos, a comparison to other endpoints measured in zebrafish under similar exposure scenarios needs to be made. For zebrafish larvae exposed to chlorpyrifos, the 48 h-LC50 was determined to be 0.39 mg/L, the 96 h-LC50 was 0.28 mg/L. After 96 h of exposure to the insecticide, the EC50 for abnormal swimming behavior of zebrafish larvae was 0.75 mg/L (Perez et al., 2013). Regarding the effects on adult zebrafish, Manjunatha and Philip (2015) observed a decrease in plasma levels of 17β-estradiol in females as well as increased plasma levels of vitellogenin and 11-ketotestosterone in males following 24–48 h of exposure to 200 μg/L chlorpyrifos. Treatments for 24 h with 35 μg/L, 88 μg/L, and 220 μg/L chlorpyrifos decreased the activity of AChE in zebrafish muscles to 29% of the control levels; but reduced swimming rates were only observed at the highest test concentration of 220 μg/L (Tilton et al., 2011a). Since the olfactory-mediated antipredator response of zebrafish was strongly disrupted at a considerably lower concentration (20 μg/L) in the present study, it proved to be a highly sensitive endpoint for assessing the toxicity of chlorpyrifos. Given the importance of the antipredator response as well as olfaction in general for the survival and fitness of fish, these findings underline the need for further research assessing behavioral and olfactory toxicity. Furthermore, the sensitivity of olfactory-mediated behaviors to chemical pollution highlights the importance of considering the inclusion of olfactory and behavioral endpoints in the risk assessment of chemicals.

4. Conclusion

In conclusion, the present study demonstrated that the three pesticides greatly differed in their effect on the antipredator response of zebrafish. Whereas fish exposed to chlorpyrifos

exhibited a clear disruption of the antipredator response, linuron appeared to rather modify only certain elements of this complex behavior. While it is known that the disruption of the antipredator response in an environmental scenario makes fish more prone to predation and decreases their chance of survival, further research is required to assess the impact of more subtle changes of this behavior. When comparing the effective concentrations of chlorpyrifos for olfaction and olfactory-mediated behavior with other endpoints, olfaction proved to be considerably more sensitive. This raises the need for future research assessing the impact of pesticides on olfaction and related behaviors as well as for the consideration of the incorporation of olfactory and behavioral endpoints into the environmental risk assessment of chemicals. For the investigation of these endpoints, zebrafish would be a well-suited model.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.124963.

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