Aquatic Toxicology 226 (2020) 105555

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Short exposure to cadmium disrupts the olfactory system of zebrafish (*Danio rerio*) – Relating altered gene expression in the olfactory organ to behavioral deficits

Sina N. Volz[a,*,1], Jonas Hausen[b,3], Milen Nachev[c], Richard Ottermanns[d], Sabrina Schiwy[a,*,2], Henner Hollert[a,2]

[a] Institute for Environmental Research, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany
[b] Core Unit for Bioinformatics Data Analysis, University of Bonn, Venusberg-Campus 1, Bonn, Germany
[c] Aquatic Ecology and Centre for Water and Environmental Research, University of Duisburg-Essen, Universitätsstrasse 5, 45141 Essen, Germany
[d] Chair of Environmental Biology and Chemodynamics, Institute for Environmental Research, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany

ARTICLE INFO

Keywords:
Behavior
Chemosensation
Metals
Olfaction
Toxicity
Zebrafish

ABSTRACT

Fish strongly rely on olfaction as a variety of essential behaviors such as foraging and predator avoidance are mediated by the olfactory system. Cadmium (Cd) is known to impair olfaction and accumulate in the olfactory epithelium (OE) and bulb (OB) of fishes. In the present study, the acute toxicity of Cd on olfaction in zebrafish (*Danio rerio*) was characterized on the molecular and behavioral level. To this end, quantitative real-time PCR was performed in order to analyze the expression of selected genes in both the OE and OB. Moreover, the response of zebrafish to an alarm cue was investigated. Following 24 h of exposure to Cd, the expression of genes associated with olfactory sensory neurons was reduced in the OE. Furthermore, the antioxidant genes peroxiredoxin 1 (*prdx1*) and heme oxygenase 1 (*hmox1*), as well as the metallothionein 2 gene (*mt2*) were upregulated in the OE, whereas *hmox1* and the stress-inducible heat shock protein 70 gene (*hsp70*) were upregulated in the OB upon exposure to Cd. Following stimulation with a conspecific skin extract, zebrafish displayed a considerable disruption of the antipredator behavior with increasing Cd concentration. Taken together, Cd impaired olfaction in zebrafish, thereby disrupting the antipredator response, which is crucial for the survival of individuals. Cellular stress followed by disruption of olfactory sensory neurons may have contributed to the observed behavioral deficits.

1. Introduction

Fish strongly rely on olfaction since essential behaviors such as foraging, predator avoidance, and the selection of a suitable mate are mediated by the olfactory system (Tierney et al., 2010). The olfactory organ of fish consists of paired rosettes, on the surface of which the olfactory epithelium (OE) is located (Byrd and Brunjes, 1995). Within the OE the olfactory sensory neurons (OSNs) are responsible for the detection of odorants (Hamdani and Døving, 2007). To date, five types of OSNs have been described, with the three major ones being ciliated, microvillous, and crypt OSNs (Byrd and Brunjes, 1995; Ahuja et al., 2014; Wakisaka et al., 2017). While ciliated OSNs detect bile salts, amino acids are perceived by microvillous OSNs (Hansen et al., 2003; Kolmakov et al., 2009; Døving et al., 2011; Dew et al., 2014). Crypt neurons respond to pheromones (Hamdani and Døving, 2006; Ahuja et al., 2013). Since OSNs are only covered by a thin layer of mucous, they are in almost direct contact with the ambient water. In such a highly exposed position, both natural odorants and contaminants can interact with them very easily (Tierney et al., 2010).

A wide variety of pollutants are known to disrupt olfaction (reviewed in Tierney et al., 2010). Being an element, cadmium (Cd) is not degradable, and thus both ubiquitously present and continuously

* Corresponding authors.
E-mail addresses: volz@biology.sdu.dk (S.N. Volz), milen.nachev@uni-due.de (M. Nachev), ottermanns@bio5.rwth-aachen.de (R. Ottermanns), schiwy@bio.uni-frankfurt.de (S. Schiwy), hollert@bio.uni-frankfurt.de (H. Hollert).
[1] Current affiliation: Department of Biology, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark.
[2] Current affiliation: Department of Evolutionary Ecology and Environmental Toxicology, Goethe University Frankfurt, Max-von-Laue-Str. 13, 60438 Frankfurt am Main, Germany.
[3] Deceased.

https://doi.org/10.1016/j.aquatox.2020.105555
Received 22 October 2019; Received in revised form 14 June 2020; Accepted 25 June 2020
Available online 29 June 2020
0166-445X/

accumulating in the environment. (Faroon et al., 2012). Point sources of Cd for environmental contamination are mining, refining, batteries, pigments, and plastics (Mendez-Armenta and Rios, 2007). In the majority of western countries, however, rock phosphate fertilizer and atmospheric deposition make up over 90 % of the anthropogenic emission (Alloway, 1995). In European stream waters, Cd concentrations range from < 0.002–1.25 µg/L and baseline values are typically below the environmental quality standards that range between 0.08 and 0.25 µg/L depending on the water hardness (European Comission, 2000; Pan et al., 2010). Yet, Cd concentrations above 1 mg/L have been measured in a few heavily polluted areas (Murphy et al., 1978; Ma et al., 2008; Wasike et al., 2019). In addition, it was shown that changes in the redox condition as well as mechanical disturbances occurring due to bioturbation (Ciutat et al., 2007; Currie et al., 2009) or flood events (Zhao and Marriott, 2013; Foulds et al., 2014; Ciszewski and Grygar, 2016) can remobilize Cd that is accumulated in sediments; thereby, Cd may partition to the water phase and become bioavailable (Audry et al., 2010; Hamzeh et al., 2014; Ciszewski and Grygar, 2016).

In fish, Cd is a non-essential metal and while low levels of it can be sequestered, cells fail to regulate higher amounts, which could subsequently lead to toxic effects (McGeer et al., 2011). Cd is known to impair the olfactory system of fish in environmentally relevant concentrations (Williams et al., 2016) and to increase prey vulnerability (Sullivan et al., 1978). Furthermore, Cd was shown to accumulate within the OE, the olfactory nerve, and the olfactory bulb (OB) of rats and fish (Evans and Hastings, 1992; Tallkvist et al., 1998).

Some research was done analyzing the effects of Cd on the OE on the molecular or cellular level; however, to the best of our knowledge, comparable investigations focusing on the OB are still missing. Moreover, while a number of studies assessed Cd-induced olfactory toxicity in fish (Matz and Krone, 2007; Wang and Gallagher, 2013; Williams and Gallagher, 2013; Dew et al., 2016; Williams et al., 2016), only a few linked molecular endpoints to adverse outcomes on the organism level. However, toxic mechanisms that occur on the molecular level may lead to deficits in the perception of cues such as alarm substances, thereby impairing the antipredator response or other olfactory-mediated behaviors (overview given by Tierney et al., 2010). Furthermore, by linking effects on the molecular level to olfactory deficits, rapidly responding molecular endpoints that are indicative of olfactory toxicity may be identified.

The aim of the present study was to characterize the acute effects of Cd on the molecular and behavioral level. Using zebrafish, we analyzed the expression of genes associated with olfaction (olfactory marker protein (*omp*), transient receptor potential cation channel, subfamily C, member 2 (*trpc2*), S100 calcium binding protein Z (*s100z*)), stress (peroxiredoxin 1 (*prdx1*), heme oxygenase 1 (*hmox1*), heat shock protein 70 (*hsp70*)), and exposure (metallothionein 2 (*mt2*)) in both the olfactory rosette and bulb. Furthermore, the antipredator response to a conspecific skin extract was investigated to link the gene expression data to behavioral changes. Collectively, we aimed at contributing to the understanding of mechanisms underlying Cd-induced impairment of olfaction and a related behavior in zebrafish.

2. Material and methods

2.1. Animals

Adult zebrafish were purchased from a local pet store and kept in the aquatic facility at RWTH Aachen University for a minimum of four weeks prior to their use in experiments. For holding water, tap water was purified by reverse osmosis and supplemented with salts (HS aqua Marin Pro Salt). The temperature was maintained at 26 ± 1 °C and animals were kept on a light:dark photoperiod of 14:10 h. Fish were fed *ad libitum* twice a day with commercial TetraMin flakes (Tetra GmbH, Melle, Germany).

2.2. Exposures

Cadmium chloride and benzocaine were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Cadmium chloride stock solutions were prepared in ultrapure water and nitric acid (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was added (0.4 % (v/v)).

Zebrafish of both sexes and minimum age of seven months were exposed to 10, 100, and 1000 µg/L Cd for 24 h. All treatment solutions were prepared with reconstituted water (demineralized water supplemented with HS aqua Marin Pro Salt); controls were reconstituted water only. Fish were exposed in either 5 L (gene expression study) or 2 L (antipredator study) of the respective treatment solution with a density of one fish per liter and under continuous aeration. Water temperature was maintained at 26 ± 1 °C. For the gene expression study, three replicate groups of five fish were exposed for each treatment; for the antipredator study, a total of twenty fish were exposed for each experimental concentration. At the beginning and end of every experiment, water samples were taken and waterborne Cd concentration, water hardness, and alkalinity were measured (please refer to supplementary material for details). 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 with 300 mg/L benzocaine followed by decapitation.

2.3. Gene expression study

2.3.1. Tissue collection, RNA isolation, and cDNA synthesis

Immediately after euthanasia, fish were dissected under a dissecting microscope. Olfactory rosettes and bulbs were removed, pooled from five fish, snap-frozen in liquid nitrogen, and stored at -80 °C until further processing. RNA isolation was performed using the NucleoSpin® RNA Kit (Macherey-Nagel, Düren, Germany) following the instructions of the manufacturer. To remove any residual genomic DNA, samples were purified employing the Invitrogen TURBO DNA-free™ Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. RNA concentrations and purity were measured with a BioDrop µLITE (BioDrop Ltd, Cambridge, UK). cDNA was synthesized from 125 ng total RNA using a random primer mix (hexamers and anchored-dT primers), a dNTP mix, and MULV Reverse Transcriptase (all purchased from New England Biolabs, Frankfurt a.M., Germany) following the manufacturer's standard protocol for first strand cDNA synthesis (NEB #M0253).

2.3.2. Quantitative real-time PCR

Primers were designed using Primer-BLAST (National Center for Biotechnology Information, Bethesda MD, USA) and NetPrimer (Premier Biosoft, Palo Alto, CA, USA) and synthesized by Eurofins Genomics (Eurofins Genomics Germany GmbH, Germany) (Table S2). For the quantitative real time PCR (qRT-PCR), cDNA was used as a template for the DNA amplification. The PCR reaction mix contained Fast SYBR™ Green Master Mix (Applied Biosystems™ by Thermo Fisher Scientific, Darmstadt, Germany), 0.5 µM of each primer, and cDNA. The qRT-PCR was conducted using a StepOnePlus™ real-time PCR system (Applied Biosystems™ by Thermo Fisher Scientific, Darmstadt, Germany). Samples were analyzed in triplicates and the DNA amplification was quantified according to Pfaffl (2001). Gene expression of the target genes was normalized to the reference gene *eef1a1l1* and expressed as fold change of the respective control values. Details are shown in the supplementary material (S2.1).

2.3.3. Data analysis

If not stated otherwise, data analyses were performed in R Version

3.5.1 (R Developmental Core Team, 2018). Three samples each consisting of either olfactory rosettes or bulbs pooled from five fish (corresponding to one replicate group) were analyzed per treatment. Expression data of individual genes were tested for homogeneity of variance and normal distribution. In case the parametric assumption was confirmed, a univariate analysis of variance (ANOVA) followed by a Dunnett's test (multcomp package) (Hothorn et al., 2008) was performed. Whenever the parametric assumption was not confirmed, a Kruskal-Wallis test followed by a Dunn's test (Dunn.test package) (Dinno, 2017) was employed. Figures were prepared using the ggplot2 package (Wickham, 2016) and cowplot package (Wilke, 2019). In addition, the expression of all selected genes in the olfactory rosette and bulb were assessed by performing Multiple Response Permutation Procedures (MRPPs) (Mielke et al., 1976) in PC-ORD (MjM Software, Gleneden Beach, Oregon) using the Euclidean distance as the distance measure.

2.4. Antipredator response study

2.4.1. Skin extract preparation

The conspecific skin extract was prepared from dead and archived control fish from previous experiments that had been snapfrozen in liquid nitrogen and stored at -80 °C until further usage. On every experimental day, fish were thawed on ice and the skin of four individuals – two males and two females (corresponds to approx. 12 cm^2) – was removed with surgical scissors and forceps. Special care was taken to ensure that no blood or other tissue was added to the skin sample. Subsequently, 60 mL of demineralized water was added, the skin was homogenized (homogenizer VDI 12, S12N-5S, VWR International GmbH, Darmstadt, Germany), and the resulting dispersion was filtered through a glass microfiber filter. The skin extract was stored on ice until usage.

2.4.2. Behavioral trials

Behavioral trials were conducted in parallel in two 35 L observation tanks filled with clear reconstituted water; no Cd was added. To prevent visual disturbances, three sides of the tanks were covered from the outside with black silicon. In addition, an opaque curtain was installed in front of the tanks. Tubing was attached to allow the delivery of stimuli to the observation tanks from behind the curtain. A camera (Rollei Actioncam 426, 4k, Rollei GmbH & Co. KG, Norderstedt, Germany) was installed in front of each tank to record fish behavior during the trial. Following 24 h of exposure to 10, 100, and 1000 µg/L Cd, fish were introduced individually into the observation tanks and left undisturbed to acclimate for 40 min. The rationale for assessing the behavior of individual fish instead of shoals was that despite of zebrafish being a shoaling fish species, it had previously been demonstrated by Mathuru et al. (2012) that the response of individuals is either comparable or stronger compared to that of small groups. Hence, by assessing the antipredator behavior of individual zebrafish we reduced the number of animals required while obtaining similar or stronger responses to conspecific skin extract. To assess whether the delivery of a solution alone induced an alarm response in control animals *via* visual or mechanical stimulation, water was employed as a blank stimulus in preliminary experiments. Skin extract was utilized to trigger the olfactory-mediated antipredator response. 5 mL of stimulus was delivered to the observation tanks *via* the tubing and the latter was flushed with an additional 5 mL of water. Between trials, observation tanks were emptied, flushed with 5 L of water, and refilled with fresh reconstituted water before the next fish were introduced.

2.4.3. Behavioral analysis

The behavior of twenty fish per treatment was tracked 7 min before and 7 min after stimulus delivery (from hereinafter referred to as pre- and post-stimulus) from the video recordings employing EthoVision® XT 11.5 (Noldus Information Technology bv, Wageningen, The Netherlands). Observation tanks were divided into a lower and upper half, and the time spent in the lower half was measured. Raw data were exported and further analyzed with R version 3.5.1 (R Developmental Core Team, 2018). Freezing (defined as motionless, except the movement of eyes and gills) and erratic movements (defined as rapid swimming bursts with multiple changes of direction) were manually analyzed from the video recordings. In order to obtain a combined assessment of all behavioral elements of the antipredator response, we used a previously developed scoring system (Volz et al., 2019) comprising the three behaviors as well as the time spent performing them.

2.4.4. Data analysis

If not stated otherwise, data analyses were performed using R Version 3.5.1 (R Developmental Core Team, 2018). To investigate whether Cd affected the behavior prior to the stimulation with the alarm substance, Mann–Whitney *U* tests were conducted comparing the individual behaviors of fish exposed to Cd to those of control animals. To verify the suitability of the selected behavioral endpoints for the assessment of the olfactory-mediated antipredator response, pre-stimulus data of bottom-dwelling, erratic movements, and freezing in control fish stimulated with either skin extract or water (blank) were tested against the respective post-stimulus values. Therefore, Wilcoxon signed-rank tests were performed. Post-stimulus data of freezing and erratic movements were corrected by subtracting the respective pre-stimulus values. Cd-induced effects on the individual behaviors associated with the antipredator response were assessed by using one-sided Mann–Whitney *U* tests. The Benjamini-Hochberg p-values adjustment was employed to correct for multiple testing. For the multivariate analysis of the antipredator response, an MRPP (Mielke et al., 1976) was performed with PC-ORD (MjM Software, Gleneden Beach, Oregon); the Euclidean distance was used as the distance measure. Figures were prepared in R using the ggplot2 package (Wickham, 2016) and cowplot package (Wilke, 2019).

3. Results

3.1. Gene expression

Exposure to Cd for 24 h resulted in a significantly decreased expression of marker genes for ciliated (*omp*) and microvillus (*trpc2*) OSNs in the olfactory rosette (Fig. 1). The expression of *s100z*, a marker for crypt neurons, increased following exposure to 10 µg/L Cd but did not significantly differ from the control level at 100 and 1000 µg/L Cd.

100 µg/L Cd induced a significant increase in the expression of the antioxidant genes *hmox1* and *prdx1* in the olfactory rosette (Fig. 2). In

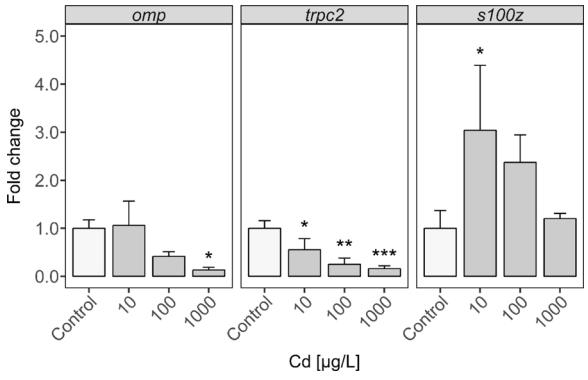

Fig. 1. Expression of OSN marker genes in the olfactory rosette following 24 h of exposure to Cd. The results are displayed as the mean fold change to the control + standard deviation. Expression levels were normalized to the reference gene *eef1a1l1*. n = 3 pools of olfactory rosettes of 5 fish. Statistically significant differences between the control and the treatments are denoted with asterisks: *p < 0.05, **p < 0.01, ***p < 0.001.

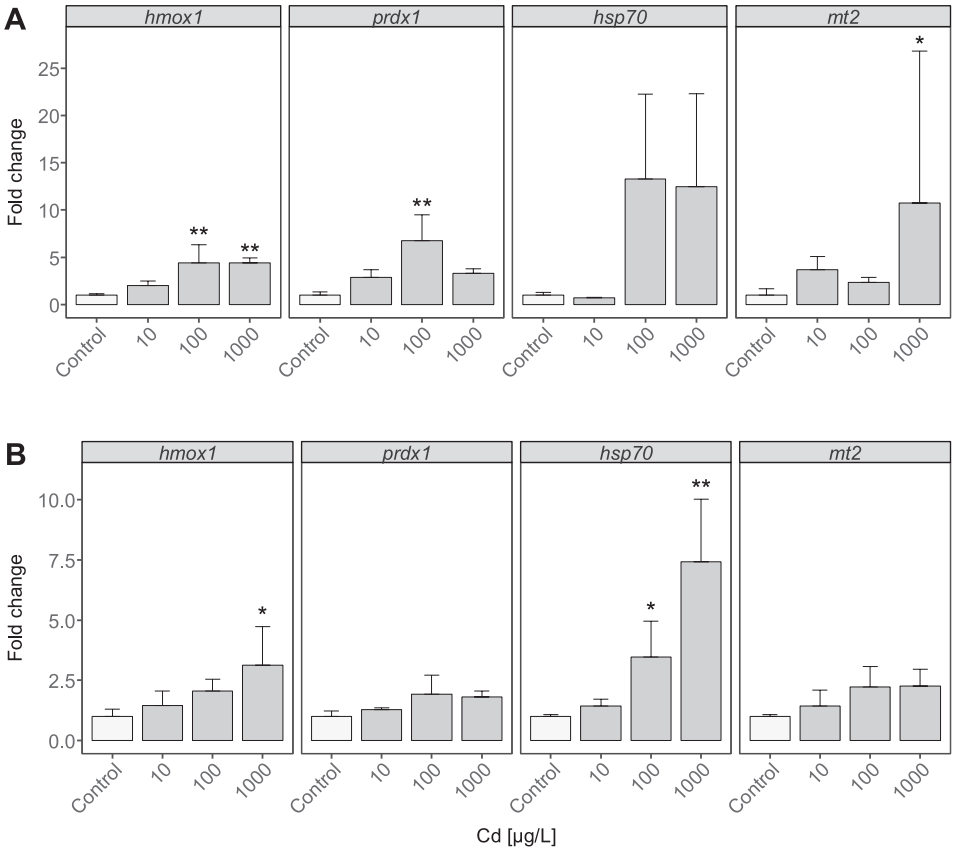

Fig. 2. Expression of selected genes in the olfactory rosette (**A**) and bulb (**B**) following 24 h of exposure to Cd. The results are displayed as the mean fold change to the control + standard deviation. Expression levels were normalized to the reference gene *eef1a1l1*. n = 3 pools of olfactory rosettes (**A**) or bulbs (**B**) of 5 fish, except for 100 µg/L Cd *hsp70*and *mt2*in the olfactory rosette, n = 2 pools of olfactory rosettes of 5 fish. Statistical significances between the control and the treatments are denoted with asterisks: *p < 0.05, **p < 0.01.

the OB, *hmox1* expression was significantly elevated in the highest test concentration and *prdx1* showed an increasing trend. While *hsp70* expression was upregulated in both the olfactory rosette and the OB, a statistically significant difference to the control only occurred for the expression in the OB. However, *hsp70* expression at 100 and 1000 µg/L Cd in the olfactory rosette was significantly induced compared to at 10 µg/L Cd (p = 0.04 and p = 0.02, respectively). Following exposure to 1000 µg/L Cd, the expression of *mt2* significantly increased in the olfactory rosette, whereas only an upward trend could be measured in the OB.

The multivariate analysis (MRPP with Euclidean distance) combining the expression of all the selected genes showed that gene expression was significantly altered in the OE in all Cd treatments (p = 0.02), whereas in the OB only 100 and 1000 µg/L Cd induced significant effects (p = 0.02).

3.2. Antipredator response

Zebrafish displayed bottom-dwelling, erratic movements, and freezing in response to the delivery of conspecific skin extract (Fig. 3). While bottom-dwelling was the most common reaction to the alarm cue, not all of the zebrafish showed erratic movements or freezing. To validate whether these behaviors are reliable endpoints for the investigation of the olfactory-mediated antipredator response, they were assessed prior and following the delivery of either conspecific skin extract as an alarm cue or water as a blank stimulus. While no changes in the selected behaviors were detected after stimulation with water, control fish significantly increased bottom-dwelling (p = 0.0005), erratic movements (p = 0.01), and freezing (p = 0.03) following the delivery of conspecific skin extract.

Prior to the addition of the conspecific skin extract, fish exposed to Cd did not display any alterations in the percentage of time spent with erratic movements or freezing. The percentage of time spent in the bottom half of the tank (bottom-dwelling), however, increased with rising Cd concentration (refer to Fig. 1 of supplementary material).

Following 24 h of exposure to 1000 µg/L Cd and delivery of conspecific skin extract, zebrafish displayed significantly less bottom-dwelling (p = 0.009). The duration of erratic movements presented a tendency to decrease after treatment with Cd. Zebrafish exposed to 10-1000 µg/L Cd displayed a concentration-dependant decline of the percentage of time spent freezing (p = 0.03 for 1000 µg/L Cd).

Since the selected behavioral elements were not independent of each other, we additionally performed multivariate data analysis. MRPP results presented a highly significant difference (p = 0.0008). Pairwise comparisons of the control with the Cd treatments showed statistically significant distinctions at 1000 µg/L Cd (p = 0.00008).

Additionally, we employed our previously developed scoring system (Volz et al., 2019), which comprises all three behaviors as well as time fish spent performing them. With increasing concentration of Cd, the score significantly declined in a concentration-dependent manner (p = 0.04 for 100 µg/L and p = 0.005 for 1000 µg/L).

4. Discussion

In the present study, Cd-induced olfactory impairment in zebrafish was evident at both the molecular and behavioral level. The assessment of the expression of genes associated with olfaction revealed a significant decrease of *omp* following 24 h of exposure to 1000 µg/L. Omp is a small cytoplasmic protein that is widely used as a marker for mature ciliated OSNs. Omp modulates cAMP kinetics in OSNs (Reisert et al., 2007), thereby ensuring the maintenance of OSN sensitivity (Nakashima et al., 2020). Reduced expression of *omp* may translate into a decreased availability of the protein and thus, impair olfactory signal transduction. Beyond that, the expression of *omp* has previously been suggested to be a sensitive marker for the loss of ciliated OSNs (Wang and Gallagher, 2013). The fact that the downregulation of *omp* in zebrafish and coho salmon (*Oncorhynchus kisutch*) after metal exposure was demonstrated to be concordant in time and concentration with a

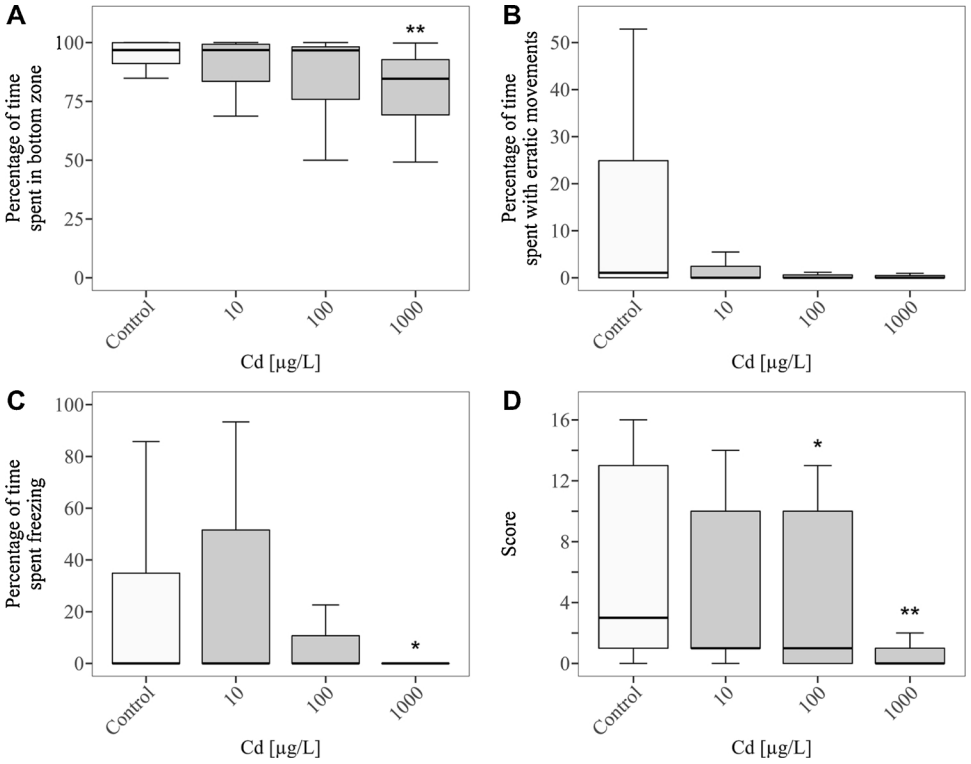

Fig. 3. Behaviors associated with the anti-predator response displayed by zebrafish following 24 h of exposure to Cd and the delivery of conspecific skin extract as an alarm cue. **A:** Percentage of time zebrafish spent with bottom-dwelling; **B:** erratic movements; **C:** freezing. **D:** Score combining all three behaviors. Bottom-dwelling 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). The sum of the score values was calculated for each fish. Statistical differences between the control and the Cd treatments are denoted with asterisks. *$p < 0.05$, **$p < 0.01$. n = 20 fish, except for 1000 µg/L Cd, n = 19 fish.

significant increase of cell death in the OE (Wang et al., 2013; Wang and Gallagher, 2013) supports this suggestion. Hence, the reduced expression of *omp* in the present study possibly indicates Cd-induced loss of ciliated OSNs; however, this hypothesis remains to be tested, for instance, by combining gene expression studies with OE histology or immunostainings employing specific antibodies against OSN marker proteins.

Transcript numbers of *trpc2* were decreased following treatment with 100 and 1000 µg/L Cd. Trpc2 is a commonly used marker protein for microvillous OSNs and was shown to have a role in the detection of amino acids (Lipschitz and Michel, 2002; Sato et al., 2005). As for *omp*, reduced expression of *trpc2* may potentially alter olfactory signal transduction. Future research should investigate whether a Cd-induced loss of microvillous OSNs is the underlying cause of the decreased expression of *trpc2*.

To assess the effect of Cd on crypt neurons, the expression of *s100z* was analyzed. Our data showed an induction of *s100z* at 10 µg/L Cd. With increasing Cd concentration, however, *s100z* expression declined and was not significantly different from control levels at 100 and 1000 µg/L. For another member of the S100 protein family, S100A2, it was proposed that this protein plays a role in the response to oxidative stress (Deshpande et al., 2000). Wang and Gallagher (2013) further demonstrated that *s100z* is likely under the control of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway, which has an important antioxidant function. Hence, one possible explanation for the upregulation of *s100z* in the present study may be the induction of an antioxidant response in the olfactory rosette by 10 µg/L Cd. Beyond that, the decrease of *s100z* expression in 100 and 1000 µg/L Cd may indicate an impact of Cd on crypt OSNs, which – as for *omp* and *trpc2*– needs to be investigated in future studies.

Previously, it was demonstrated that oxidative stress likely is a critical factor in the impairment of olfaction by metals (Wang and Gallagher, 2013). The generation of reactive oxygen species (ROS) is a common mode of action of different metals and can affect various cellular components, such as proteins, DNA, and lipids, thereby modulating their function (Manca et al., 1991; Mendez-Armenta et al., 2003; Lopez et al., 2006). Moreover, ROS act as signaling molecules in the induction of apoptosis (Othumpangat et al., 2005). Studies with zebrafish larvae demonstrated that 3 h of exposure to 2.81 and 14 mg/L Cd, respectively, induced cell death in the olfactory placode and disrupted olfactory-mediated behaviors (Blechinger et al., 2007; Wang and Gallagher, 2013). Since the enzymes Prdx1 and Hmox1 hold important antioxidant functions (Novoselov et al., 1999; Ryter and Choi, 2002), their gene expression is used as a biomarker for oxidative and cellular stress (Williams and Gallagher, 2013; Williams et al., 2016). After 48 h of treatment with 347 µg/L Cd, the induction of *hmox1* expression coincided with histological effects, such as decreased number of OSNs and increased number of apoptotic cells (Williams and Gallagher, 2013). Our data showed induction of *hmox1* and *prdx1* in the olfactory rosette after treatment with 100 µg/L Cd which is indicative of an antioxidant response. This is consistent with observations in the OE of coho salmon following 24 h of exposure to 347 µg/L (Williams and Gallagher, 2013) and in 120 hpf zebrafish larvae upon 24 h of treatment with 117 µg/L Cd (Heffern et al., 2018).

Heat-shock proteins, also called stress-proteins, are part of the primary defense mechanisms coping with proteotoxicity. Hsp70 is one of the most widely studied stress-proteins and its gene expression is highly inducible by a wide variety of environmental contaminants (reviewed in Bierkens, 2000). Zebrafish embryos exposed to Cd for up to 96 h displayed an upregulation of *hsp70* in the OE (Matz and Krone, 2007). Furthermore, whole larvae expression of *hsp70* increased after 24 h of treatment with 117 µg/L Cd (Heffern et al., 2018). In the study at hand, *hsp70* expression increased at 100 and 1000 µg/L Cd compared to 10 µg/L, suggesting cellular stress and the occurrence of protein denaturation in the olfactory rosette. Alterations in the expression of *mt2* were generally comparable to those for *hsp70*, but, they were slightly less pronounced. Metallothioneins are small cytosolic metal-binding proteins and their gene expression is commonly used as a marker for metal exposure. Hence, the elevation in the expression of *mt2* is consistent with the induction of this gene by Cd.

The investigation of the expression of the same genes in the OB showed similar trends; however, measured fold changes in mRNA transcript levels were lower in the OB compared to in the olfactory rosettes. While *hmox1* and *hsp70* were significantly upregulated, slight

upward trends could be observed for *prdx1* and *mt2*. Taken together, the overall trend of the mRNA data suggests that Cd induces an antioxidant response in the OB. Previous studies have shown that Cd entered the olfactory system and accumulated in the OE, the olfactory nerve, and the OB of fishes (Evans and Hastings, 1992; Tallkvist et al., 1998, 2002; Scott et al., 2003). Complexation with metallothionein likely facilitates this process (Nishimura et al., 1992; Tallkvist et al., 2002). Since metallothioneins are thought to play a vital role in the detoxification of metals by sequestration, Cd is unlikely to exert any toxic effects as long as it is bound to them. Nonetheless, our gene expression data suggested that an antioxidant response occurred in the olfactory organ, proposing that Cd was likely also present in its free and toxic form as well.

The antipredator response of fishes belonging to the Ostariophysi superorder is one example of such an olfactory-mediated behavior. The ostariophysans make up approximately 64 % of all freshwater fish. Their common feature is the presence of specialized epidermal club cells, which contain the alarm pheromone (Frisch, 1938; Smith, 1992). Upon injury, this pheromone is released and can be detected by conspecifics, thereby eliciting a typical fright response. Following the delivery of conspecific skin extract, control fish in the present study significantly increased the time spent in the bottom half of the tank as well as the time spent freezing and displaying erratic movements. These behaviors are typical parts of the antipredator response of zebrafish (Gerlai, 2010). The lack of a change in these behaviors when delivering water as a blank stimulus instead of the alarm cue further showed that these behaviors were not induced by the mechanical or visual stimulus due to the addition of the solution. Thus, bottom-dwelling, erratic movements, and freezing are suitable endpoints for assessing the olfactory-mediated antipredator response.

Zebrafish displaying erratic movements often performed these close to the bottom of the tank. Considering the natural environment of these small freshwater cyprinids, this behavior would stir up sediment and debris at the ground, thereby making it harder for predators to spot and catch their prey (Speedie and Gerlai, 2008). After exposure to Cd, the duration of erratic movements showed a downward trend, suggesting a disruption of this behavior. Following treatment with Cd, both the number of fish exhibiting freezing behavior and the duration of freezing decreased substantially. Freezing in most cases was the final stage of the antipredator response of zebrafish and was only performed when fish appeared to be in severe distress. In agreement with these findings, a study assessing anxiety in zebrafish using a dark/bright preference test found that freezing was the strongest measure of this emotional state (Blaser et al., 2010). Hence, freezing was assigned the highest score value in our previously developed scoring system (Volz et al., 2019). The results of utilizing the scoring system in the present study demonstrated a clear concentration-dependent decrease of the score with rising Cd concentration. Together with the outcome of the multivariate analysis, this leads to the conclusion that Cd altered the olfactory-mediated antipredator response of zebrafish. For rainbow trout, it was shown that exposure to 2 µg/L Cd for seven days disrupted antipredator behavior, whereas shorter exposure times or lower concentrations had no effect (Scott et al., 2003). Furthermore, it was demonstrated that coho salmon exposed to 347 µg/L Cd for 48 h displayed a reduction of the antipredator response that coincided with a decreased number of OSNs in the OE (Williams and Gallagher, 2013). Developmental exposure to 20 µg/ L Cd for 50 days disrupted the antipredator response of zebrafish and the effect was still present following 14 days of depuration (Kusch et al., 2008). The occurrence of behavioral deficits at lower Cd concentrations compared to the present study is probably due to the considerably longer exposure duration that likely resulted in the accumulation of Cd in the olfactory system (Tallkvist et al., 2002; Williams et al., 2016). In addition, a higher sensitivity of the early life stages compared to adult zebrafish to Cd-induced olfactory deficits could explain the difference in effective concentrations. However, Matz and Krone (2007) did not observe an impairment of olfactory-mediated behavior following 96 h exposure of zebrafish larvae to 56 µg/L Cd. In the environment, impairment of the antipredator response would substantially increase the likelihood of falling prey to a predator, thereby decreasing the chance of survival for the individual (McIntyre et al., 2012). While an impairment, injury, or loss of OSNs could explain the disruption of the antipredator behavior observed in the present study, we can not exclude other causes. Monaco et al. (2016 and 2017) observed alterations in the neuroglia as well as neurodegeneration in the brain of zebrafish following 16 days of exposure to Cd. Previously, it was shown that the medial bundle of the medial olfactory tract projecting to the medial pallium of the forebrain (Nikonov et al., 2005) is involved in the antipredator response (Hamdani et al., 2000; Mathuru, 2008). Hence, Cd-induced alterations in the forebrain as described by Monaco et al. (2016 and 2017) may also cause the impairment of antipredator behavior. Yet, since neurodegeneration in adult zebrafish brains was only evident following 16 days and not after 2 or 7 days of treatment with Cd (Monaco et al., 2017), impairment or loss of OSNs appear to be more likely causes of the observed behavioral effects.

Cd-induced olfactory toxicity occurred at concentrations that were considerably higher (factor 10–1000) as typically measured in surface waters (Pan et al., 2010). In some exceptional cases, maximal Cd concentrations exceeding 10 µg/L were reported, for instance, 13.5 µg/L in filtered and 513 µg/L in unfiltered water of the Tarapaya River in Bolivia (Smolders et al., 2003), 610 µg/L in surface water in the Cerbat Mountains in Arizona (Rösner, 1998), and 1 mg/L in the River Kuywa in Kenya (Wasike et al., 2019). Cd is known to accumulate in sediments and may be remobilized due to changes in the redox potential or mechanical disturbances, for example occurring during flood events; this may lead to temporarily increased waterborne Cd concentrations (Audry et al., 2010; Hamzeh et al., 2014; Ciszewski and Grygar, 2016) that could approach concentrations which were shown to induce an effect in the study at hand. Besides, Cd was demonstrated to accumulate within the olfactory rosette and bulb of fish and rats (Gottofrey and Tjalve, 1991; Tallkvist et al., 2002); hence, prolonged exposure may lead to substantially higher internal Cd concentrations. In fact, following 48 h of exposure to up to 30 µg/L Cd, coho salmon displayed no disruption of their aversive response to L-cysteine; however, when treated with 0.3 µg/L Cd for 16 days, the aversion response was significantly altered (Williams et al., 2016). Consequently, chronic exposure to low and environmentally relevant concentrations may result in effects comparable to those observed in the present study after acute exposure to high concentrations of Cd.

As recently pointed out by Legradi et al. (2018), the increasing numbers of potentially neurotoxic contaminants in the environment pose great risks for humans and animals alike and raise the need of implementing the investigation of “eco-neurotoxicity” into the environmental risk assessment of chemicals. Given the importance of olfactory function for a variety of behaviors pivotal for fish, such as the antipredator response assessed in the present study, the inclusion of olfactory endpoints into an “eco-neurotoxicity” testing strategy should be considered. In this context, the expression of OSN marker genes (*omp*, *trpc2*, *s100z*), as well as the genes associated with stress (*hmox1*, *prdx1*, *hsp70*) and exposure (*mt2*), may be useful biomarkers. While this study showed that alterations in the expression of these genes coincided with impairment of olfactory-mediated behavior, future research is required to establish the missing link between gene expression and physiological markers of cellular stress and OSN injury. Moreover, thresholds need to be determined, the exceedance of which results in an effect relevant to olfactory function. Besides, additional genes that may be suitable endpoints for olfactory toxicity ought to be identified and it should be tested whether an array of these genes would reliably indicate the toxic potential of a variety of different substances to the olfactory system.

In conclusion, this study demonstrated that Cd disrupted the olfactory system of zebrafish at the molecular and behavioral level. In the olfactory rosette, Cd induced an antioxidant response and altered the

expression of OSN marker genes; these results are consistent with those of previously published studies. Furthermore, this study was the first to show that Cd further induced the expression of genes related to cellular stress in the OB. Observed effects on olfactory-mediated behavior were concordant in time and concentration with altered expression of antioxidant as well as OSN marker genes; thus, a causal relationship between these two endpoints is possible. However, additional research is required to fill in the remaining knowledge gaps, for instance, by combining the assessment of antioxidant and OSN marker gene expression with the measurement of lipid peroxidation in the olfactory organ and histological or immunohistochemical analysis of OSN damage. The assessment of Cd-induced effects in an environmentally relevant (long-term) exposure scenario was beyond the scope of the present study and exposure concentrations were considerably higher than commonly found in the environment. However, since our results suggested that Cd was rapidly taken up into the olfactory rosette and bulb of zebrafish, an accumulation of this metal in the olfactory tissue is to be expected. Consequently, even significantly lower concentrations of Cd in the environment may lead to high amounts of the metal within the olfactory system; these could ultimately overwhelm cellular defense mechanisms, thereby disrupting olfaction. Given the importance of the olfactory system for almost every aspect of a fish's life, olfactory deficits may threaten both individuals and populations.

Funding

This work was supported by the Deutsche Bundesstiftung Umwelt DBU(German Federal Environmental Foundation), Germany, with a personal scholarship granted to S. N. Volz.

CRediT authorship contribution statement

Sina N. Volz: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization. **Jonas Hausen:** Software, Validation, Formal analysis, Writing - review & editing. **Milen Nachev:** Investigation, Writing - review & editing. **Richard Ottermanns:** Formal analysis, Writing - review & editing. **Sabrina Schiwy:** Conceptualization, Writing - review & editing, Supervision. **Henner Hollert:** Conceptualization, Resources, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors thank the Deutsche Bundesstiftung Umwelt DBU (German Federal Environmental Foundation) for a personal scholarship granted to S. N. Volz. We kindly thank Sarah Johann, Milena Esser, and Simone Hotz for helping maintain the zebrafish used in this study. Furthermore, we express our gratitude to Nadine Volz, for the linguistic editing of this manuscript. The authors thank Noldus Information Technology bv and Tecan Group Ltd. for their contribution to this study as a partner of the Students Lab "Fascinating Environment" at Aachen Biology and Biotechnology (ABBt).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2020.105555.

References

Ahuja, G., Ivandić, I., Saltürk, M., Oka, Y., Nadler, W., Korsching, S.I., 2013. Zebrafish crypt neurons project to a single, identified mediodorsal glomerulus. Sci. Rep.-UK 3, 2063. https://doi.org/10.1038/srep02063.

Ahuja, G., Nia, S.B., Zapilko, V., Shiriagin, V., Kowatschew, D., Oka, Y., Korsching, S.I., 2014. Kappe neurons, a novel population of olfactory sensory neurons. Sci. Rep. 4, 4037. https://doi.org/10.1038/srep04037.

Alloway, B.J., 1995. Chapter 6, Cadmium. Heavy Metals in Soils. Chapman and Hall, London, pp. 122–151.

Audry, S., Grosbois, C., Bril, H., Schäfer, J., Kierczak, J., Blanc, G., 2010. Post-depositional redistribution of trace metals in reservoir sediments of a mining/smelting-impacted watershed (the Lot River, SW France). Appl. Geochem. 25, 778–794. https://doi.org/10.1016/j.apgeochem.2010.02.009.

Bierkens, J.G., 2000. Applications and pitfalls of stress-proteins in biomonitoring. Toxicology 153, 61–72.

Blaser, R.E., Chadwick, L., McGinnis, G.C., 2010. Behavioral measures of anxiety in zebrafish (Danio rerio). Behav. Brain Res. 208, 56–62. https://doi.org/10.1016/j.bbr.2009.11.009.

Blechinger, S.R., Kusch, R.C., Haugo, K., Matz, C., Chivers, D.P., Krone, P.H., 2007. Brief embryonic cadmium exposure induces a stress response and cell death in the developing olfactory system followed by long-term olfactory deficits in juvenile zebrafish. Toxicol. Appl. Pharmacol. 224, 72–80. https://doi.org/10.1016/j.taap.2007.06.025.

Byrd, C.A., Brunjes, P.C., 1995. Organization of the olfactory system in the adult zebrafish: histological, immunohistochemical, and quantitative analysis. J. Comp. Neurol. 358, 247–259. https://doi.org/10.1002/cne.903580207.

Ciszewski, D., Grygar, T.M., 2016. A review of flood-related storage and remobilization of heavy metal pollutants in river systems. Water Air Soil Pollut. 227, 239. https://doi.org/10.1007/s11270-016-2934-8.

Ciutat, A., Gerino, M., Boudou, A., 2007. Remobilization and bioavailability of cadmium from historically contaminated sediments: influence of bioturbation by tubificids. Ecotoxicol. Environ. Saf. 68, 108–117. https://doi.org/10.1016/j.ecoenv.2006.06.011.

Currie, R.S., Fairchild, W.L., Muir, D.C.G., 2009. Remobilization and export of cadmium from lake sediments by emerging insects. Environ. Toxicol. Chem. 16, 2333–2338. https://doi.org/10.1002/etc.5620161119.

Deshpande, R., Woods, T.L., Fu, J., Zhang, T., Stoll, S.W., Elder, J.T., 2000. Biochemical characterization of S100A2 in human keratinocytes: subcellular localization, dimerization, and oxidative cross-linking1. J. Invest. Dermatol. 115, 477–485. https://doi.org/10.1046/j.1523-1747.2000.00078.x.

Dew, W.A., Azizishirazi, A., Pyle, G.G., 2014. Contaminant-specific targeting of olfactory sensory neuron classes: connecting neuron class impairment with behavioural deficits. Chemosphere. https://doi.org/10.1016/j.chemosphere.2014.02.047.

Dew, W.A., Veldhoen, N., Carew, A.C., Helbing, C.C., Pyle, G.G., 2016. Cadmium-induced olfactory dysfunction in rainbow trout: effects of binary and quaternary metal mixtures. Aquat. Toxicol. 172, 86–94. https://doi.org/10.1016/j.aquatox.2015.12.018.

Dinno, A., 2017. dunn.test: Dunn's Test of Multiple Comparisons Using Rank Sums. from. https://CRAN.R-project.org/package=dunn.test.

Døving, K.B., Hansson, K.-A., Backström, T., Hamdani, E.H., 2011. Visualizing a set of olfactory sensory neurons responding to a bile salt. J. Exp. Biol. 214, 80–87. https://doi.org/10.1242/jeb.046607.

European Comission, 2000. Directive 2000/60/EC of the European Parliament and of the Council, Establishing a Framework for Community Action in the Field of Water Policy.

Evans, J., Hastings, L., 1992. Accumulation of Cd(II) in the CNS depending on the route of administration: intraperitoneal, intratracheal, or intranasal. Toxicol. Sci. 19, 275–278. https://doi.org/10.1093/toxsci/19.2.275.

Faroon, Obaid, Ashizawa, Annette, Wright, Scott, Tucker, Pam, Jenkins, Kim, Ingerman, Lisa, Rudisill, Catherine, 2012. Toxicological Profile for Cadmium. Agency for Toxic Substances and Disease Regstry (ATSDR), Atlanta (GA).

Foulds, S.A., Brewer, P.A., Macklin, M.G., Haresign, W., Betson, R.E., Rassner, S.M.E., 2014. Flood-related contamination in catchments affected by historical metal mining: an unexpected and emerging hazard of climate change. Sci. Total Environ. 476–477, 165–180. https://doi.org/10.1016/j.scitotenv.2013.12.079.

Frisch, K.V., 1938. Zur Psychologie des Fisch-Schwarmes. Naturwissenschaften 26, 601–606. https://doi.org/10.1007/BF01590598.

Gerlai, R., 2010. Zebrafish antipredatory responses: a future for translational research? Behav. Brain Res. 207, 223–231. https://doi.org/10.1016/j.bbr.2009.10.008.

Gottofrey, J., Tjalve, H., 1991. Axonal transport of cadmium in the olfactory nerve of the pike. Pharmacol. Toxicol. 69, 242–252.

Hamdani, E.H., Døving, K.B., 2006. Specific projection of the sensory crypt cells in the olfactory system in Crucian Carp, Carassius carassius. Chem. Senses 31, 63–67. https://doi.org/10.1093/chemse/bjj006.

Hamdani, E.H., Døving, K.B., 2007. The functional organization of the fish olfactory system. Prog. Neurobiol. 82, 80–86. https://doi.org/10.1016/j.pneurobio.2007.02.007.

Hamdani, E.H., Stabell, O.B., Alexander, G., Doving, K.B., 2000. Alarm reaction in the crucian carp is mediated by the medial bundle of the medial olfactory tract. Chem. Senses 25, 103–109. https://doi.org/10.1093/chemse/25.1.103.

Hamzeh, M., Ouddane, B., Daye, M., Halwani, J., 2014. Trace metal mobilization from surficial sediments of the Seine River Estuary. Water Air Soil Pollut. 225, 1878. https://doi.org/10.1007/s11270-014-1878-0.

Hansen, A., Rolen, S.H., Anderson, K., Morita, Y., Caprio, J., Finger, T.E., 2003. Correlation between olfactory receptor cell type and function in the channel catfish. J. Neurosci. 23, 9328–9339.

Heffern, K., Tierney, K., Gallagher, E.P., 2018. Comparative effects of cadmium, zinc, arsenic and chromium on olfactory-mediated neurobehavior and gene expression in larval zebrafish (Danio rerio). Aquat. Toxicol. 201, 83–90. https://doi.org/10.1016/j.aquatox.2018.05.016.

Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biom. J. 50, 17.
Kolmakov, N.N., Hubbard, P.C., Lopes, O., Canario, A.V.M., 2009. Effect of acute copper sulfate exposure on olfactory responses to amino acids and pheromones in goldfish (Carassius auratus). Environ. Sci. Technol. 43, 8393–8399. https://doi.org/10.1021/es901166m.
Kusch, R.C., Krone, P.H., Chivers, D.P., 2008. Chronic exposure to low concentrations of waterborne cadmium during embryonic and larval development results in the long-term hindrance of antipredator behavior in zebrafish. Environ. Toxicol. Chem. 27, 705–710. https://doi.org/10.1897/07-273.1.
Legradi, J.B., Di Paolo, C., Kraak, M.H.S., van der Geest, H.G., Schymanski, E.L., Williams, A.J., Dingemans, M.M.L., Massei, R., Brack, W., Cousin, X., Begout, M.L., van der Oost, R., Carion, A., Suarez-Ulloa, V., Silvestre, F., Escher, B.I., Engwall, M., Nilén, G., Keiter, S.H., Pollet, D., Waldmann, P., Kienle, C., Werner, I., Haigis, A.C., Knapen, D., Vergauwen, L., Spehr, M., Schulz, W., Busch, W., Leuthold, D., Scholz, S., vom Berg, C.M., Basu, N., Murphy, C.A., Lampert, A., Kuckelkorn, J., Grummt, T., Hollert, H., 2018. An ecotoxicological view on neurotoxicity assessment. ESEU 30, 46. https://doi.org/10.1186/s12302-018-0173-x.
Lipschitz, D., Michel, W., 2002. Amino acid odorants stimulate microvillar sensory neurons. Chem. Senses 27, 277–286. https://doi.org/10.1093/chemse/27.3.277.
Lopez, E., Arce, C., Oset-Gasque, M.J., Canadas, S., Gonzalez, M.P., 2006. Cadmium induces reactive oxygen species generation and lipid peroxidation in cortical neurons in culture. Free Radic. Biol. Med. 40, 940–951. https://doi.org/10.1016/j.freeradbiomed.2005.10.062.
Ma, W., Wang, L., He, Y., Yan, Y., 2008. Tissue-specific cadmium and metallothionein levels in freshwater crab Sinopotamon henanense during acute exposure to waterborne cadmium. Environ. Toxicol. 23, 393–400. https://doi.org/10.1002/tox.20339.
Manca, D., Ricard, A.C., Trottier, B., Chevalier, G., 1991. Studies on lipid peroxidation in rat tissues following administration of low and moderate doses of cadmium chloride. Toxicology 67, 303–323.
Mathuru, A.S., 2008. The alarm response in Zebrafish: innate fear in a vertebrate genetic model AU - Jesuthasan, Suresh J. J. Neurogenet. 22, 211–228. https://doi.org/10.1080/01677060802298475.
Mathuru, A.S., Kibat, C., Cheong, W.F., Shui, G., Wenk, M.R., Friedrich, R.W., Jesuthasan, S., 2012. Chondroitin fragments are odorants that trigger fear behavior in fish. Curr. Biol. 22, 538–544. https://doi.org/10.1016/j.cub.2012.01.061.
Matz, C.J., Krone, P.H., 2007. Cell death, stress-responsive transgene activation, and deficits in the olfactory system of larval zebrafish following cadmium exposure. Environ. Sci. Technol. 41, 5143–5148.
McGeer, J.C., Niyogi, S., Scott Smith, D., 2011. In: In: Wood, C.M., Farrell, A.P., Brauner, C.J. (Eds.), Cadmium. Fish Physiology 31. Academic Press, pp. 125–184. https://doi.org/10.1016/S1546-5098(11)31025-4.
McIntyre, J.K., Baldwin, D.H., Beauchamp, D.A., Scholz, N.L., 2012. Low-level copper exposures increase visibility and vulnerability of juvenile coho salmon to cutthroat trout predators. Ecol. Appl. 22, 1460–1471. https://doi.org/10.1890/11-2001.1.
Mendez-Armenta, M., Rios, C., 2007. Cadmium neurotoxicity. Environ. Toxicol. Pharmacol. 23, 350–358. https://doi.org/10.1016/j.etap.2006.11.009.
Mendez-Armenta, M., Villeda-Hernandez, J., Barroso-Moguel, R., Nava-Ruiz, C., Jimenez-Capdeville, M.E., Rios, C., 2003. Brain regional lipid peroxidation and metallothionein levels of developing rats exposed to cadmium and dexamethasone. Toxicol. Lett. 144, 151–157.
Mielke, P.W., Berry, K.J., Johnson, E.S., 1976. Multi-response permutation procedures for a priori classifications. Commun. Stat.-Theor. M 5 1409–1424. https://doi.org/10.1080/03610927608827451.
Monaco, A., Grimaldi, M.C., Ferrandino, I., 2016. Neuroglial alterations in the zebrafish brain exposed to cadmium chloride. J. Appl. Toxicol. 36, 1629–1638. https://doi.org/10.1002/jat.3328.
Monaco, A., Capriello, T., Grimaldi, M.C., Schiano, V., Ferrandino, I., 2017. Neurodegeneration in zebrafish embryos and adults after cadmium exposure. Eur. J. Histochem. 61https://doi.org/10.4081/ejh.2017.2833. 2833-2833.
Murphy, B.R., Atchison, G.J., McIntosh, A.W., Kolar, D.J., 1978. Cadmium and zinc content of fish from an industrially contaminated lake. J. Fish Biol. 13, 327–335. https://doi.org/10.1111/j.1095-8649.1978.tb03441.x.
Nakashima, N., Nakashima, K., Taura, A., Takaku-Nakashima, A., Ohmori, H., Takano, M., 2020. Olfactory marker protein directly buffers cAMP to avoid depolarization-induced silencing of olfactory receptor neurons. Nat. Commun. 11, 2188. https://doi.org/10.1038/s41467-020-15917-2.
Nikonov, A.A., Finger, T.E., Caprio, J., 2005. Beyond the olfactory bulb: an odotopic map in the forebrain. Proc. Natl. Acad. Sci. U. S. A. 102, 18688–18693. https://doi.org/10.1073/pnas.0505241102.
Nishimura, N., Nishimura, H., Ghaffar, A., Tohyama, C., 1992. Localization of metallothionein in the brain of rat and mouse. J. Histochem. Cytochem. 40, 309–315. https://doi.org/10.1177/40.2.1552172.
Novoselov, S.V., Peshenko, I.V., Popov, V.I., Novoselov, V.I., Bystrova, M.F., Evdokimov, V.J., Kamzalov, S.S., Merkulova, M.I., Shuvaeva, T.M., Lipkin, V.M., Fesenko, E.E., 1999. Localization of 28-kDa peroxiredoxin in rat epithelial tissues and its antioxidant properties. Cell Tissue Res. 298, 471–480.
Othumpangat, S., Kashon, M., Joseph, P., 2005. Eukaryotic translation initiation factor 4E is a cellular target for toxicity and death due to exposure to cadmium chloride. J. Biol. Chem. 280, 25162–25169. https://doi.org/10.1074/jbc.M414303200.
Pan, J., Plant, J.A., Voulvoulis, N., Oates, C.J., Ihlenfeld, C., 2010. Cadmium levels in Europe: implications for human health. Environ. Geochem. Health 32, 1–12. https://doi.org/10.1007/s10653-009-9273-2.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.
R Developmental Core Team, 2018. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Reisert, J., Yau, K.W., Margolis, F.L., 2007. Olfactory marker protein modulates the cAMP kinetics of the odour-induced response in cilia of mouse olfactory receptor neurons. J. Physiol. 585, 731–740. https://doi.org/10.1113/jphysiol.2007.142471.
Rösner, U., 1998. Effects of historical mining activities on surface water and groundwater - an example from northwest Arizona. Environ. Geol. 33, 224–230. https://doi.org/10.1007/s002540050241.
Ryter, S.W., Choi, A.M., 2002. Heme oxygenase-1: molecular mechanisms of gene expression in oxygen-related stress. Antioxid. Redox Signal. 4, 625–632. https://doi.org/10.1089/15230860260220120.
Sato, Y., Miyasaka, N., Yoshihara, Y., 2005. Mutually exclusive glomerular innervation by two distinct types of olfactory sensory neurons revealed in transgenic zebrafish. J. Neurosci. 25, 4889–4897. https://doi.org/10.1523/JNEUROSCI.0679-05.2005.
Scott, G.R., Sloman, K.A., Rouleau, C., Wood, C.M., 2003. Cadmium disrupts behavioural and physiological responses to alarm substance in juvenile rainbow trout (Oncorhynchus mykiss). J. Exp. Biol. 206, 1779–1790. https://doi.org/10.1242/jeb.00353.
Smith, R.J.F., 1992. Alarm signals in fishes. Rev. Fish Biol. Fish. 2, 33–63. https://doi.org/10.1007/bf00042916.
Smolders, A.J.P., Lock, R.A.C., Van der Velde, G., Medina Hoyos, R.I., Roelofs, J.G.M., 2003. Effects of Mining Activities on Heavy Metal Concentrations in Water, Sediment, and Macroinvertebrates in Different Reaches of the Pilcomayo River, South America. Arch. Environ. Contam. Toxicol. 44, 0314–0323. https://doi.org/10.1007/s00244-002-2042-1.
Speedie, N., Gerlai, R., 2008. "Alarm substance induced behavioral responses in zebrafish (Danio rerio). Behav. Brain Res. 188, 168–177. https://doi.org/10.1016/j.bbr.2007.10.031.
Sullivan, J.F., Atchison, G.J., Kolar, D.J., McIntosh, A.W., 1978. Changes in the predator–prey behavior of fathead minnows (Pimephales promelas) and largemouth bass (Micropterus salmoides) caused by cadmium. J. Fish Res. Board Can. 35, 446–451. https://doi.org/10.1139/f78-077.
Tallkvist, J., Henriksson, J., d'Argy, R., Tjalve, H., 1998. Transport and subcellular distribution of nickel in the olfactory system of pikes and rats. Toxicol. Sci. 43, 196–203. https://doi.org/10.1006/toxs.1998.2438.
Tallkvist, J., Persson, E., Henriksson, J., Tjalve, H., 2002. Cadmium-metallothionein interactions in the olfactory pathways of rats and pikes. Toxicol. Sci. 67, 108–113.
Tierney, K.B., Baldwin, D.H., Hara, T.J., Ross, P.S., Scholz, N.L., Kennedy, C.J., 2010. Olfactory toxicity in fishes. Aquat. Toxicol. 96, 2–26. https://doi.org/10.1016/j.aquatox.2009.09.019.
Volz, S.N., Hausen, J., Smith, K., Ottermanns, R., Schaeffer, A., Schiwy, S., Hollert, H., 2019. Do you smell the danger? Effects of three commonly used pesticides on the olfactory-mediated antipredator response of zebrafish (Danio rerio). Chemosphere 124963. https://doi.org/10.1016/j.chemosphere.2019.124963.
Wakisaka, N., Miyasaka, N., Koide, T., Masuda, M., Hiraki-Kajiyama, T., Yoshihara, Y., 2017. An Adenosine receptor for olfaction in fish. Curr. Biol. 27 (1437–1447), e1434. https://doi.org/10.1016/j.cub.2017.04.014.
Wang, L., Gallagher, E.P., 2013. Role of Nrf2 antioxidant defense in mitigating cadmium-induced oxidative stress in the olfactory system of zebrafish. Toxicol. Appl. Pharmacol. 266, 177–186. https://doi.org/10.1016/j.taap.2012.11.010.
Wang, L., Espinoza, H.M., Gallagher, E.P., 2013. Brief exposure to copper induces apoptosis and alters mediators of olfactory signal transduction in coho salmon. Chemosphere 93, 2639–2643. https://doi.org/10.1016/j.chemosphere.2013.08.044.
Wasike, P.W., Nawiri, M.P., Wanyonyi, A.A., 2019. Levels of heavy metals (Pb, Mn, Cu and Cd) in water from River Kuywa and the adjacent wells. Environ. Ecol. Res. 7.3, 135–138. https://doi.org/10.13189/eer.2019.070303.
Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York.
Wilke, C.O., 2019. cowplot: Streamlined Plot Theme and Plot Annotations for' ggplot2'.
Williams, C.R., Gallagher, E.P., 2013. Effects of cadmium on olfactory mediated behaviors and molecular biomarkers in coho salmon (Oncorhynchus kisutch). Aquat. Toxicol. 140–141, 295–302. https://doi.org/10.1016/j.aquatox.2013.06.010.
Williams, C.R., MacDonald, J.W., Bammler, T.K., Paulsen, M.H., Simpson, C.D., Gallagher, E.P., 2016. Cadmium exposure differentially alters odorant-driven behaviors and expression of olfactory receptors in juvenile coho salmon (Oncorhynchus kisutch). Toxicol. Sci. 154, 267–277. https://doi.org/10.1093/toxsci/kfw172.
Zhao, Y., Marriott, S.B., 2013. Dispersion and remobilisation of heavy metals in the river severn system, UK. Procedia Environ. Sci. 18, 167–173. https://doi.org/10.1016/j.proenv.2013.04.022.