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Brief report

Death-associated odors induce stress in zebrafish

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ABSTRACT

Living animals exploit information released from dead animals to conduct adaptive biological responses. For instance, a recently published study has shown that avoidance behavior is triggered by death-associated odors in zebrafish. Stress can clearly act as an adaptive response that allows an organism to deal with an imminent threat. However, it has not been demonstrated whether these chemical cues are stressful for fish. Here, we confirmed that dead zebrafish scents induce defensive behavior in live conspecifics. Additionally, we show for the first time in fish that these scents increase cortisol in conspecifics. To reach this conclusion, firstly, we exposed zebrafish to multi-sensorial cues (*e.g.*, visual, tactile, chemical cues) from dead conspecifics that displayed defensive behaviors and increased cortisol. Also, when we limited zebrafish to chemical cues from dead conspecifics, similar responses arose. These responses coincide with the decaying destruction of epidermal cells, indicating that defensive and stress responses could take place as an effect of substances emanating from decaying flesh, as well as alarm substance released due to rupture of epidermal cells. Taken together, these results illustrate that living zebrafish utilize cues from dead conspecific to avoid or to cope with danger and ensure survival.

Introduction

Living animals exploit information released from dead animals to conduct adaptive biological responses. In fish (sea lampreys), an extract from the total carcass was verified to act as a repellent to live conspecifics (Wagner et al., 2011), although a specific substance has not been identified. In zebrafish, the diamines (putrescine and cadaverine) that emanate from decaying flesh were found to induce an odor-driven defensive behavior (Hussain et al., 2013).

Some chemical cues that are linked to defensive responses are able to induce stress in fish. Alarm substances, released due to the rupture of epidermis club cells (Rehnberg and Schreck, 1987; Rehnberg et al., 1987; Toa et al., 2004), and disturbance substances, released when fish are exposed to an aversive condition (Barcellos et al., 2011; Oliveira et al., 2013), induce conspecifics' stress response by causing an increase in cortisol levels. It stands to reason that death-associated odors could cause physiological stress in fish, but this hypothesis has not yet been tested. Here, for the first time in fish, we show that dead zebrafish scents induce a whole-body cortisol increase in live conspecifics.

Methods

Fish

A population of 1500 mixed-sex, adult wild-type zebrafish (*Danio rerio*) was held under a natural photoperiod (approximately 14 h L: 10 h D). The water was maintained at 28.0 ± 2.0 °C, with a pH of 7.0 ± 0.6, dissolved oxygen content of 6.8 ± 0.4 mg/L, total ammonia content of <0.01 mg/L and a total hardness and alkalinity of 6 and 22 mg/L of $CaCO_3$, respectively. To avoid the introduction of undesirable chemical factors during the experiments, the aquaria were not cleaned, the water was not changed, and the fish were not fed. All experimental procedures were approved by the Institutional Ethics Committee at the University of Passo Fundo (protocol number 03/2012).

Experimental design

General consideration

In the experiments below, we subjected groups of zebrafish (10 fish per aquarium — 30 cm × 30 cm × 30 cm, 25 L of water volume) to cues

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(multi-sensorial or chemical cues only) from dead conspecifics and measured typical defensive behaviors (time near the bottom and freezing) and whole-body cortisol levels.

Experiment 1. Reactions to multi-sensorial cues emanating from dead conspecifics.

After 3 h in aquaria, the groups of zebrafish were directly exposed to 2 size-matched conspecifics immediately after death. The defensive behaviors and cortisol levels in these exposed fish were assessed either immediately or at 2 h intervals for up to 12 h after the introduction of the dead fish, with unexposed fish used as a control. This setup was replicated 3 times with additional zebrafish per time point (0 to 12 h) and stimulus (presence of dead fish or control condition) totaling 21 independent zebrafish batches. For whole-body cortisol determination, pools of 2 fish (to obtain approximately 0.5 g of tissue) were examined, with a total of 8 pools of 2 fish for each treatment and time point.

Experiment 2. Exposure to water from aquaria containing dead fish.

We investigated the effect of water-born cues from size-matched, dead zebrafish on the whole-body cortisol response. In 4 independent conditions, zebrafish were exposed to 1) dead conspecifics at 10 h post-death, 2) water from 10 h post-death conspecifics, 3) water from live conspecifics or 4) distilled water. Groups of ten live zebrafish were maintained in each glass receiver aquarium (30 cm × 30 cm × 30 cm; 25 L). Size-matched conspecifics were killed by concussion and allocated to donor aquaria for 10 h (2 fish/donor). Each receiver aquarium was connected to only one donor, and the conditioned water was always transferred toward the receiver. Water was transferred from the donor to the receiver aquarium for 15 min, and fish behavior was registered for 5 min before the exposed fish were killed by severing their spinal cord for subsequent cortisol analysis.

Water was transferred through a pipe by gravity (80 mL per min) and reached the receiver aquarium through an outlet in a corner situated 1.75 cm from the bottom and 1.75 cm from the wall. The donor container was not refilled during the experiment to avoid dilution of the released chemical substances. In pre-trial experiments, we tested the system by adding methylene blue to the donor containers and observed that after 20 min of water transfer, transmittance to the receiver aquaria was equivalent to 88% of that in the respective donor. No visual contact was allowed between the donor and receiver aquaria. We used a dark curtain surrounding the whole aquarium system to prevent undesirable disturbances. Manipulation of the water flow was performed outside of this area to avoid handling interference.

Procedures and techniques

Cortisol extraction and analysis

In each group, 20 fish were used at each sampling time, forming 10 pooled samples of 2 fish each. Tissue cortisol levels were used as an indicator of the stress response. Fish were captured, immediately frozen in liquid nitrogen for 10–30 s and then stored at −20 °C until cortisol extraction. To prevent a possible handling-induced stress response, the time period between capture and killing was <30 s.

Whole-body cortisol was extracted using the method described by Sink et al. (2007). Each fish was weighed, minced and placed in a disposable Stomacher blender bag with 2 mL of phosphate-buffered saline (PBS, pH 7.4) for 6 min. The contents were then transferred to 5 mL of laboratory-grade ethyl ether in a 10-mL screw top disposable test tube. This solution was vortexed for 1 min, followed by centrifugation for 10 min at 3000 rpm, and it was then immediately frozen in liquid nitrogen. The unfrozen portion (ethyl ether containing cortisol) was decanted and transferred to a new tube and completely evaporated under a gentle stream of nitrogen for 2 h, yielding a lipid extract containing cortisol, which was then stored at −20 °C.

The accuracy of cortisol detection was tested by calculating the recovery from samples spiked with known amounts of cortisol (50, 25 and 12.5 ng/mL). The mean detection accuracy for the spiked samples was 94.3%. All cortisol values were adjusted based on the observed recovery using the following equation: cortisol value = measured value × 1.0604.

The tissue extracts were resuspended in 1 mL of PBS, and whole-body cortisol levels were measured in duplicate samples of each extract using a commercially available ELISA kit (EIAgen™ CORTISOL test, BioChem ImmunoSystems). This kit was fully validated for zebrafish tissue extracts using the methodology proposed by Sink et al. (2007). The accuracy was tested by calculating the recovery in samples spiked with known amounts of cortisol. The precision was tested by calculating the intra-assay coefficient of variation (CV) of 12 repeated assays in 7 randomly chosen samples on the same plate, and reproducibility was tested by assaying the same samples on different plates and calculating the inter-assay CV. To test for linearity and parallelism, serial dilutions of tissue extracts were performed in the buffer provided with the kit. We detected a strong positive correlation between the curves ($R^2 = 0.8918$) and determined that the samples displayed low inter- and intra-assay CVs (7–10% and 5–9%, respectively).

Behavior quantification

The water column was divided into 4 areas of equal size, from the bottom to the surface. The time that fish spent in the bottom area and the number of freezing fish were observed and manually recorded, and the % of the session time for each of these behaviors was computed later on. The rationale for quantifying fish behavior via observation and manual registration was based on the findings of Speedie and Gerlai (2008), which clearly show that zebrafish responses to alarm substances can be reliably quantified by visual-manual recording as well as through computerized video tracking methods.

We quantified freezing and time spent near the tank bottom as indicators of defensive reactions (Gerlai and Csányi, 1990; Gerlai et al., 2000). The duration of these behaviors was expressed as a percentage of the total observation session length. The onset of freezing was considered when at least 3 out of the 10 zebrafish exhibited complete immobility, with movement only in the gills and/or the eyes (Speedie and Gerlai, 2008), and the freezing behavior ended when at least 3 fish stopped exhibiting immobility.

Histological analysis

To detect histological changes in the skin of dead zebrafish and the frequency of damaged/ruptured club and mucus epidermis cells at 0, 2, 4, 6, 8, 10 and 12 h after death, 42 zebrafish killed prior to aquaria introduction were stored in 5 mL of 10% buffered formalin. Whole fish were dehydrated through a graded ethanol series and then cleared in xylene and embedded in paraffin. Sections with a thickness of 5–6 μm were prepared from paraffin blocks using a Reichert microtome. These sections were then stained with hematoxylin–eosin and examined under a Leica DME100 light microscope.

Statistics

The Kolmogorov–Smirnov test revealed that the data were normally distributed. However, the Levene test revealed that some data were heteroscedastic. When homoscedasticity was observed, we conducted one-way or two-way ANOVA, according to the number of independent factors, and complemented the ANOVAs with the Newman–Keuls test. In these cases, statistically significant differences were assumed at $P < 0.05$. ANOVA is robust to variance heterogeneity in large, balanced study designs. Thus, in cases where the data were heteroscedastic, the raw data were used in the model. In this case, we complemented the ANOVA with the Scheffé test, which is a more conservative post-hoc test, and we used a conservative level of significance ($P < 0.01$, see Underwood, 1997). Moreover,