Stress responses to conspecific visual cues of predation risk in zebrafish

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Chemical communication of predation risk is a common trait in fish. Prey fish under risk of predation can signal the risk to conspecific fish that display defensive reactions. Fish also assess predation risk by visual cues and change behavior accordingly. Here, we wonder if these behavioral changes act as visual alarm signal to conspecific fish that are not initially under risk. We show that shoals of zebrafish visually exposed to a predator display antipredator behaviors. In addition, these defensive maneuvers trigger antipredator in conspecific and, concomitantly, stimulatethe hypothalamus-pituitary-interrenal axis, leading to cortisol increase.Thus, herein, we show that zebrafish defensive behaviors act as visual alarm cues that induce antipredator and stress response in conspecific fish.

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43 **Abstract**

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1. Introduction

Prey-predator interaction occurs throughout the animal kingdom (Cresswel, 2010) with peculiar and general characteristics in every interaction. We can say that this interaction occurs in every direction (prey to predator, predator to prey, prey to prey and even predator to predator) (Barcellos et al., 2014, Mullan et al., 2015; Dunlop-Hayden & Rehage, 2011). The prey can access the predator trough a diversity of signals that can be visual, olfactory, acoustic, vibration, (Barcellos et al., 2014, Barreto et al., 2003). In fish, we have a vast literature citing these types of perceptions (Wisendem et al., 2004, Barcellos et al., 2011) of a predator or even of a conspecific fish treated by a predator (Jordão, 2000). The different combination of this different signals of the presence of a predator, or even about diverse ways of communication about predator threatening, between conspecifics prey, have different effects in anti-predator maneuvers (O`Connor et al., 2015).

During prey-predator interaction, the early detection of a predation risk is crucial for prey survival (Allen, 1975) and chemical communication plays a key role on risk assessment (Chivers & Smith, 1998). Prey fish display antipredator behaviors when perceive the odor of a predator (direct perception; *e.g.* Miyai et al., 2016) or when are alerted by chemical cues released by other preys (indirect perception; *e.g.* Barcellos et al., 2011 and 2014; Barreto et al., 2013;Oliveira et al., 2014). Regarding chemical communication of risk of predation, there is a huge body of evidences, although there are still many unanswered questions. On the other hand, prey fish also detect predators by visual cues and act properly with defensive reactions to deal with these threats (Kalluef et al., 2014), but, in this case, it is far less explored in the literature.

The visual presence of a predator induce changes in prey behavior and physiology (Barreto et al., 2003; Gerlai, 2003; Barcellos et al., 2007; Miller & Gerlai, 2007; Gebauer et al., 2011). As

a diurnal fish species, we can cite zebrafish, who clearly uses this sensorial modality for communication with conspecifics. Eavesdroping is one type of visual communication, wherein individual use the available visual public information based on conspecific fish behaviors (Abril-de-Abreu et al., 2017). These visual cues allow them to evaluate the context and adjust their behavior appropriately. The zebrafish are able to change their behavior via eavesdropping (Abril-de-Abreu et al., 2017).

In this context, we suppose that predator-induced behavioral changes could act as a visual alarm signal that provoke defensive reactions in conspecific fish. Herein, we show in zebrafish (*Danio rerio*) that antipredator behavior is a visual alarm cue for conspecific zebrafish unexposed to a predator that induces defensive maneuvers and, also, act as a stressor that induce a cortisol surge.

2. Materials and Methods

2.1. Ethical note

This study was approved at protocol #20/2016, by the Ethics Commission for Animal Use of Universidade de Passo Fundo (Passo Fundo, RS, Brazil) and all methods were carried out in accordance with the guidelines of National Council of Animal Experimentation Control (CONCEA).

2.2. Zebrafish and housing conditions

Wild-type zebrafish (*Danio rerio*), adults (± 8 months), both sexes, with an average of ±5 cm and ± 0.4 g), were maintained under a photoperiod of ~ 14 h L/10 h D in indoor holding tanks (2 fish/L). The water was maintained as follows: 28.0 ± 2.0 °C; pH of 7.0 ± 0.6; dissolved oxygen at 6.8 ± 0.4 mg/L; total ammonia at <0.01 mg/L; total hardness at 6 mg/L; and alkalinity at 22

mg/L of $CaCO_3$. The fish were fed twice a day (09:00 h and 16:00 h), until satiation, with commercial flakes (TetraMin®, Tetra, Melle, Germany). The dimension of the aquarium of stock is 13 x 30 x 40 cm (length, width and height), total water volume of 13 L and the stocking density similar to holding tank of 2 fish/L. The experimental chambers have the dimension of 40x30x30 (length, width and height) replete with 30 L of water and the stock density in this was 1 fish per 3 liters.

All experiments were conducted in quadruplicate. Ten zebrafish were used to form the sender (SF) and 10 to form receiver (RF) fish shoals in respectively groups: control, predator, and non-predator situations. The same was done for additional controls with 10 fish for predator treatment and 10 fish for non-predator. During the experiment, we used a total of 300 zebrafish for analyses.

2.3. Experimental design and procedures

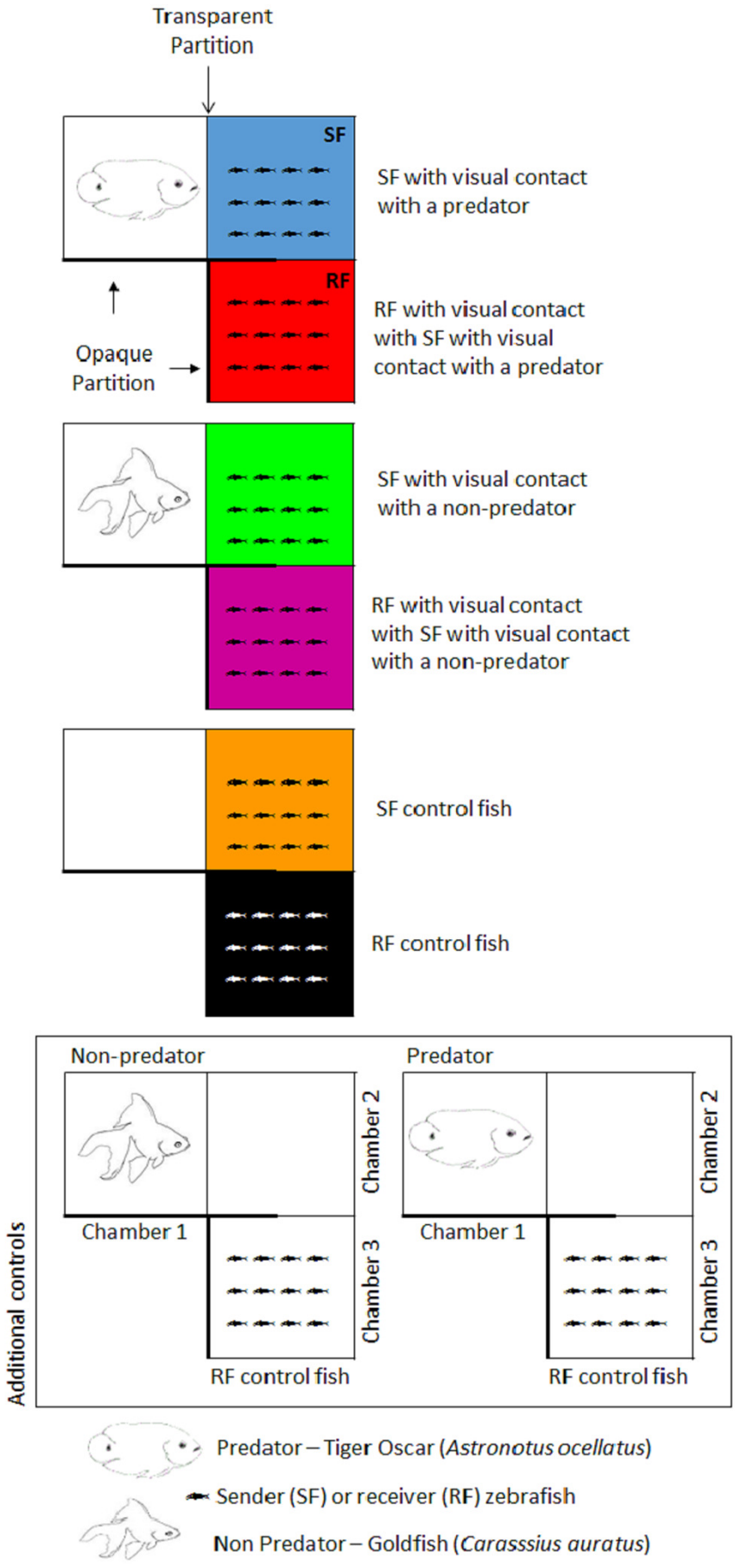
As our study strategy, we kept zebrafish shoal in a chamber that allowed visualization of a predator or non-predator fish, or a tank with only water. This condition was called (hereafter) as sender fish (SF, Fig. 1). In an adjacent chamber, we placed other zebrafish shoals, named as receiver fish (RF, Fig. 1), that permitted only the visualization of the SF shoal.

The three chambers were completely sealed to avoid any water/chemical communication between chambers. Therefore, we set up three experimental conditions. In the first condition, RF was evaluated during SF exposure to the view of a predator (the tiger oscar *Astronotus ocellatus*). In the second condition, we used a harmless fish (the goldfish, *Carassius auratus*) to test whether the effect of the view of any fish produced behavioral changes in SF and, consequently, in RF. In

the last condition, SF was exposed to the view of an aquarium with water but without any fish, controlling lab-handling processes.

Moreover, we conducted two additional control conditions (Fig. 1) to show that RF are not capable of seeing directly the predator, two groups of receiver fishes were evaluated isolated (without the visual cues emanating from conspecific fish), one staying in their chamber 3, while the predator stayed in his respective chamber 1. Another group of receiver fish stayed in their chamber 3, while the non-predator stayed in his respective chamber 1. We set up a sample size of n = 10/ each condition. Initially, SF and RF zebrafish shoals and stimulus fish (non-predator and predator) were introduced in their respective chambers to an adaptation period of 24h. In this period, SF fish could not visualize the stimulus chamber (predator fish, harmless fish, or tank with only water) and RF could visualize SF during all adaptation and experiment period. Visual contact was blocked using an opaque plastic plaque. After this adaptation period, the plaque was removed allowing the visualization between stimulus chamber and SF for 60 min, integrally video recorded for behavioral analysis. After the visualization period, both SF and RF fish were captured, killed by immediate spinal section, frozen in liquid nitrogen and stored at -20 ^{o}C until required to whole-body cortisol assay.

Chambers were always completely cleaned before a new replicate was set up. During the experiments, the aquaria were not cleaned, the water was not changed, and the fish were not feed to avoid the effects of handling procedures, because fish stayed into the chambers only for 24 h. We guaranteed that no exchange of water occurred between each chamber. We previously filled the chambers with water individually and observed that the adjacent chamber remained totally dry. With this procedure, we ensured that the communication between fish of each chamber occurred just via visual sensory.


Transparent Partition
SF
SF with visual contact with a predator
RF
RF with visual contact with SF with visual contact with a predator
Opaque Partition
SF with visual contact with a non-predator
RF with visual contact with SF with visual contact with a non-predator
SF control fish
RF control fish
Non-predator
Predator
Chamber 1
Chamber 2
Chamber 3
Additional controls
RF control fish
Predator – Tiger Oscar (Astronotus ocellatus)
Sender (SF) or receiver (RF) zebrafish
Non Predator – Goldfish (Carasssius auratus)

148 **Figure 1**.Schematic representation of the experimental conditions.The fish drawings in the
149 graphics were drawn by LB.
150
151 ***2.4. Cortisol extraction and determination***
152 To extract and determine the whole-body cortisol we used 4 to 6 fish of each experimental
153 group of each replicate, totalizing 16-24 fish. There is a variation among cortisol "n" samples
154 because whole-body cortisol analysis requires a minimum of 0.5g of tissue extract, so since fish
155 were small, we used pooled samples of two or three fish, aiming to attain the final "n" number of
156 8 samples.
157 Tissue cortisol levels were used as an indicator of the stress response. To prevent a possible
158 handling-induced stress response, the time period between capture and killing was < 30 s. Each
159 fish was weighed, minced and placed in a tube containing 3 ml of phosphate buffered saline (PBSg,
160 pH 7.3). The contents were then transferred to a tube containing ethyl ether and subjected to
161 vortexing for then centrifuged after immediately frozen in liquid nitrogen. The thawed portion
162 (cortisol containing diethyl ether) was decanted and transferred to a new tube and evaporated
163 completely, to yield a lipid extract containing cortisol, which was then stored at - 20°C.
164 The tissue extracts were resuspended in PBSg and the whole-body cortisol levels were
165 measured using a commercially available ELISA kit (EIAgen ™ cortisol test,
166 BioChemImmunosystems). This kit is fully validated for zebrafish tissue extracts using the
167 methodology proposed by Sink et al. (2007). The accuracy was tested by calculating the recovery
168 in samples spiked with known amounts of cortisol. The precision was tested by calculating the
169 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 = 0.892) and determined that the samples displayed low inter- and intra-assay CVs (7–10 % and 5–9 %, respectively).

2.5. Behavioral quantification

We repeated the methodology employed in Oliveira et al. (2014). Briefly, the water column was divided into three areas of equal size, from the bottom to the surface. The time that fish spent in the bottom area was observed and manually recorded by a single experienced observer without knowing what group was being assessed, and the % of the session time for each of these behaviors was calculated later on. The rationale for quantifying fish behavior via observation and manual registration was based on the findings of Speedie & 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 the time spent near the tank bottom as an indicator of defensive reactions (Gerlai & Csányi, 1990; Gerlai et al., 2000; Quadros et al., 2016). The duration of this behavior was expressed as a percentage of the total observation session duration. The onset of the time at tank bottom was considered when at least 3 out of the 10 zebrafish remain in the bottom area based on previous method and data (Speedie & Gerlai, 2008).

2.6. Statistics

For whole-body cortisol and behavior values, we compare all treatments and also proceeded two specific comparisons between zebrafish senders and between receiver zebrafish of

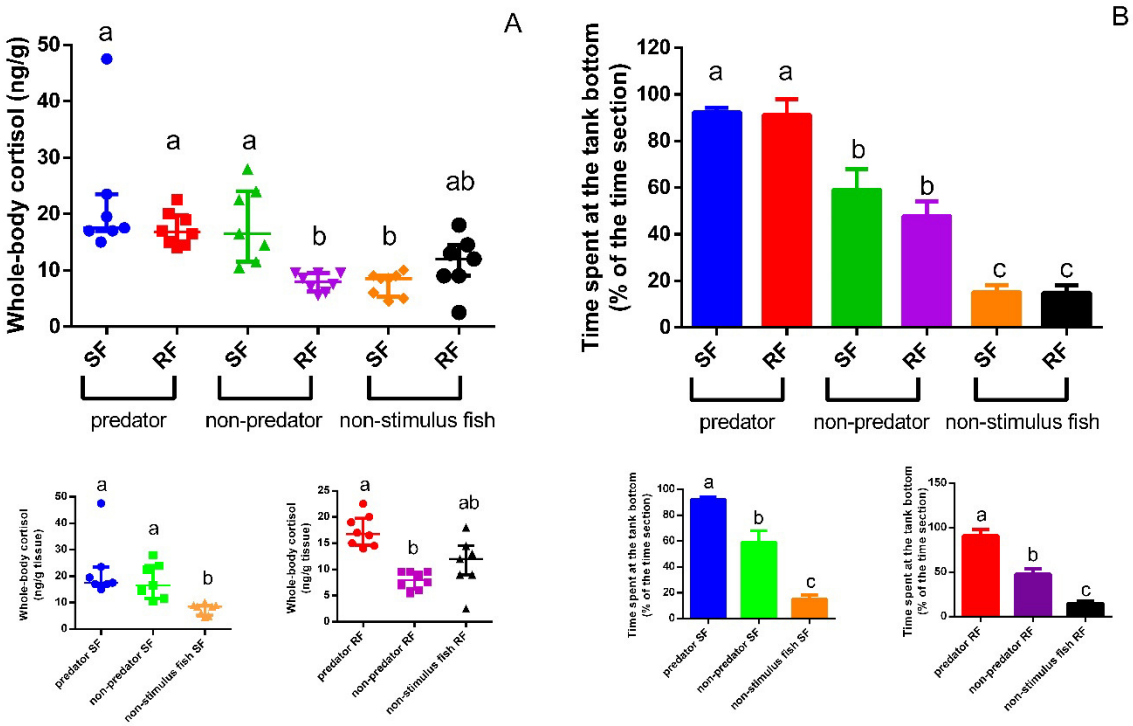
193 experimental conditions. Regarding cortisol values, a Kolmogorov-Smirnov test showed that the
194 samples were derived from populations that did not follow normal distributions. A Bartlett test
195 indicated that the SDs of the samples in the same experiment were statistically indistinguishable.
196 Therefore, we applied the Kruskal-Wallis test followed by Dunn's multiple comparison test.
197 Regarding time spent at the bottom aquaria, the data passed in Kolmogorov-Smirnov test and
198 Bartlett test and, therefore, we applied an one-way ANOVA, followed by Tukey's multiple
199 comparison test to compare the means in each experiment. Significant differences were set at $\alpha =$
200 0.05.

201 **3. Results**

202 We found that visual perception of the predator increased whole-body cortisol in the SF
203 and RFs in relation SF control ($P<0.0001$, $K=30.68$, SF comparison $P=0.0006$, $K=14.92$ and RF
204 comparison $P=0.001$, $K=13.74$). This effect did not occur for SF and RF in the non-predator fish
205 treatment, in which only SF increased cortisol in relation to the SF control. The tank with only
206 water elicited no significant cortisol response (Figure 2A).

207 Regarding defensive behavior, for fish exposed to the predator, both SF and RF had
208 augmented the time spent close to the tank bottom. In fish exposed to a non-predator fish, this
209 variable was also increased for SF and RF, but this response was less intense (statistically lower)
210 than those SF and RF observed in predator exposure treatment, considering the comparison of both
211 groups (non-predator and predator treatment) with the control group (non-stimulus fish). The
212 aquaria with only water induced no significant change in the defensive behavior (Figure 2B,
213 $P<0.0001$, $F_{5,18}=38.66$, SF comparison $P<0.0001$, $F_{2,9}=50.17$ and RF comparison $P<0.0001$,
214 $F_{5,18}=46.63$). Zebrafish from the two additional controls presented very low cortisol concentrations

215 (2.88 ± 0.74 and 3.63 ± 0.59 ng/g tissue), typically found in non-stimulated control fish (Barcellos
216 et al., 2007; 2014).



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219 **Figure 2**. Whole-body cortisol response (A) and time spent in the tank bottom (B) of SF and RF.
220 Data were expressed as median (± interquartile range) in the panel A and as mean (± S.E.M.) in
221 the panel B. Data were compared by Kruskal-Wallis complemented by a Dunn's Multiple
222 Comparisons Test (panel A) and by one-way Anova followed by Tukey´s multiple range test (n =
223 7 – 8 for cortisol and 4 for behavioral analysis). Different letters above medians or means indicate
224 the statistical difference.

225

226 **4. Discussion**

We showed that zebrafish visually exposed to predator display antipredator behaviors. that in turn acted as visual alarm cue for conspecific fish. Zebrafish, unexposed to a predator, showed defensive maneuvers when watched their conspecific displaying defensive behaviors. In addition, these visual cues induced activation of hypothalamus-pituitary-interrenal axis (stress response), culminating with a whole-body cortisol increase. These endocrine and behavioral responses might be associated with stress events, like predation risk, since they are well described in the biological interaction between prey and predator in some species (Barton 2002; Clinchy et al., 2011; Archard at al., 2012). Based on our data, we can affirm that eavesdropping occurs via visual sensory system in zebrafish facing their conspecifics in a context of predation risk. Zebrafish in a direct visual contact with a harmless fish, that had an interpretation of a false predation risk, initiated a short-term alteration in their behavior and an irreversible stress response. Here we named an irreversible stress response because we postulated that this cortisol response once triggered shows a typical increase in cortisol levels, that we realized for this group of fish, whereas the behavioral response can be quickly adjusted to the moment. Contrastingly, their conspecifics observing their altered behavior had the capability of interpret and process the information as a non-dangerous situation, evidenced by the lack of alterations in behavioral and endocrine stress responses in receiver fish.

Sender and Receiver fish presented elevated cortisol in response to a context where a predator was visually present, hence, a condition of potential imminent predation risk. When in visual contact with a non-predator or with an empty tank, this cortisol increase was not observed in receiver fish. Another interesting result and a response described as an anti-predator behavior (Gerlai, 2003) is the time spent near to the tank bottom, which increased in sender fish when in visual contact whit a predator and also in their respective receiver fish. We can affirm that zebrafish just viewing the behavior of their conspecifics reacting to the predator presence, is able to interpret

as a predation imminent risk, and triggers an anti-predatory response. These results indicate a complex form of communication in zebrafish when dealing with predators because further the direct visualizing of the predator induce defensive and stress responses, this risk can be communicated and stress conspecifics that are not seeing a predator, an indirect response to a risk of a predator attack. It indicates the occurrence of a net of communication based on visual cue that can lead shoals of zebrafish deal with predators. It could take place by a chain reaction when a single fish detect a predator and respond to this threat. Consequently, the defensive behavior propagates throughout the entire shoal. Based on the same logic, a shoal displaying anti-predator responses can induce fish in another shoal to do the same and so on. It is plausible because, in a shoal of fish, copying is a behavior that has been observed: it is common, for instance, the leader of a shoal to "command" the group during foraging navigation (Reebs, 2000). The same can occur during defensive reactions and this hypothesis deserves future investigations.

The increased whole-body cortisol in zebrafish when in visual contact with a predator was previously described (Barcellos et al., 2007). Surprisingly, zebrafish with a visual contact with the non-predator fish, presented a similar increase in cortisol levels. We can highlight that the cortisol response for this group (SF-non-predator treatment) was also a response of smaller magnitude compared to zebrafish viewing a predator. Nonetheless, receiver fish viewing these conspecifics had no elevated cortisol. The cortisol value of SF exposed to non-predator fish was higher, but the behaviour (time in the tank bottom) was similar. Thus, since RF receive only visual cues, this similar behaviour cannot elicit a cortisol response. In the non-predator treatment, both sender and receiver fish had an augmented time spent near the tank bottom when compared with the control group (without a stimulus fish). However, this response was smaller when compared with the predator exposure treatment.

We affirm that the anti-predator response showed for fish directly visualizing the predator (Sender fish) was a determinant factor for triggering an anti-predatory behavior in receiver ones, confirming that communication of a threat occurred by visual cues in a context of an interpretation of anti-predator behavior displayed by sender fishes. The unexpected behavioral and stress response in non-predator treatment may be due to the limitations assessment of the non-predator fish, since zebrafish have only visual contact with it. The recognition by fish of a real predation risk when it is not completely obvious might depend on upon also the perception of other cues, such as fish odor (Korpi&Wisenden, 2001) or mechanical cues (Hegab & Wei, 2014). A visual recognition of a predator by the prey fish is based on movement characteristics of the predator (Barcellos et al., 2007). Since our non-predator goldfish was very active, zebrafish might be momently interpreted as a predator. In our experiment, fish received only visual stimulus. The absence of the combination of different cues may be induced a 'misinterpretation' of a harmless stimulus by zebrafish. This supposed 'misinterpretation' of predation risk would have caused the observed cortisol increase in sender fish. Interestingly, zebrafish viewing their conspecific that view a non-predator did not alter their behaviour and did not trigger a cortisol response. This suggests that the explanation of 'misinterpretation' might make sense.

Our findings also highlight the importance to be careful in relation to visual cues in zebrafish, mainly in those who will be used in experiments. Others visual stimulus, (*e.g.* humans presence, other fish species or even other animals) can be interpreted like a threatening cue, having effects on the behavioral and hormonal patterns in zebrafish and, consequently influencing experimental results. Thus, our study brings a better comprehension about the predator-prey interaction and the communication along conspecifics submitted to a predator threatening in aquatic environment.

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