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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Abstract

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.

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 eveasdropping (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 (*Daniorerio*), 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), *ad libitum*, 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 triplicate. 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 240 zebrafish for analyses.

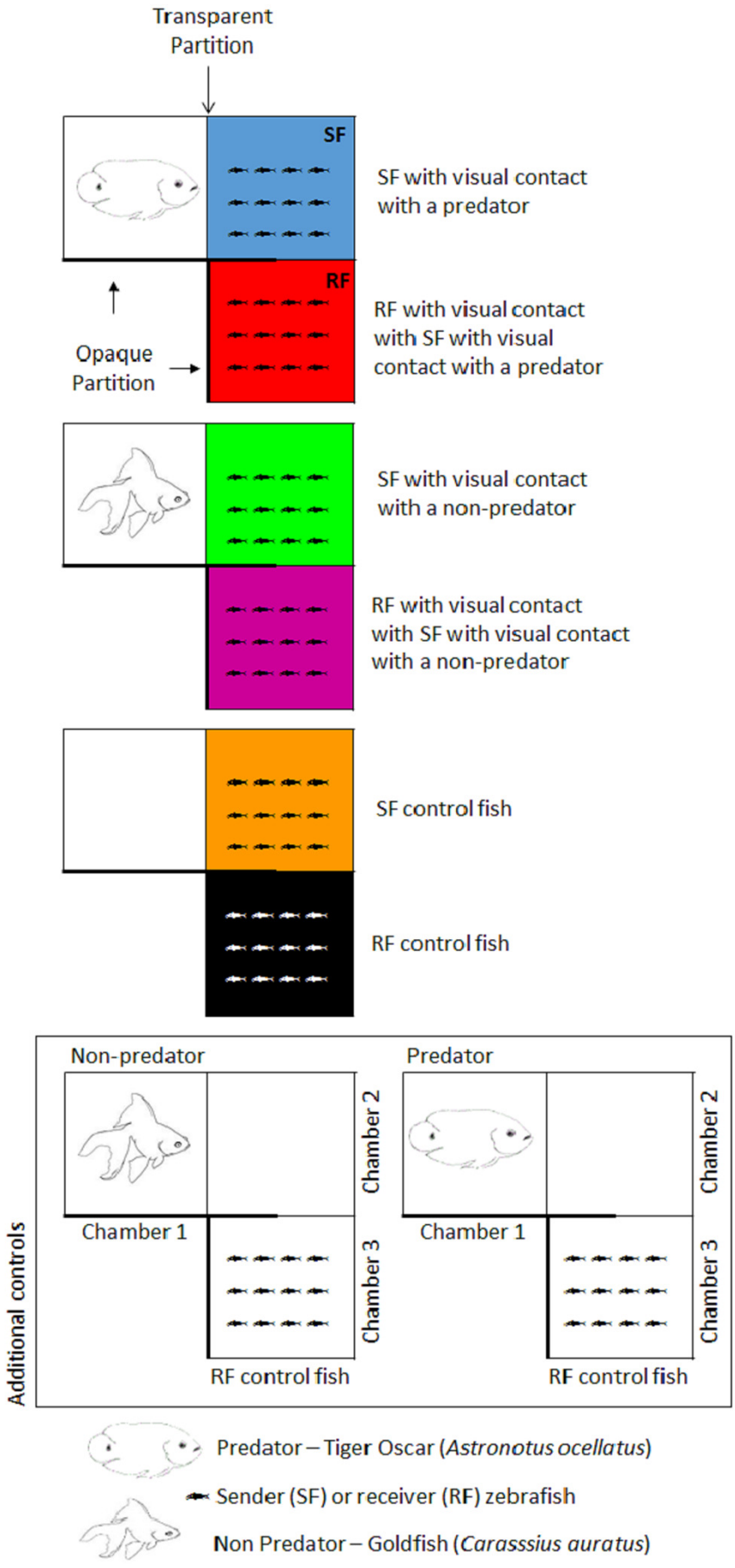
2.3. Experimental design and procedures

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.

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

138 Chambers were always completely cleaned before a new replicate was set up. During the
139 experiments, the aquaria were not cleaned, the water was not changed, and the fish were not fedto
140 avoid the effects of handling procedures, because fish stayed into the chambers only for 24 h. We
141 guaranteed that no exchange of water occurred between each chamber.We previously filled the
142 chambers with water individually and observed that the adjacent chamber remained totally dry.
143 With this procedure, we ensured that the communication between fish of each chamber occurred
144 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
RF control fish
Predator – Tiger Oscar (Astronotus ocellatus)
Sender (SF) or receiver (RF) zebrafish
Non Predator – Goldfish (Carasssius auratus)

146 **Figure 1**.Schematic representation of the experimental conditions.The fish drawings in the
147 graphics were drawn by LB.

148 ***2.4. Cortisol extraction and determination***

149 To extract and determine the whole-body cortisol we used all 10 fish of each experimental
150 group of each replicate, totalizing 30 fish. There is a variation among cortisol "n" samples because
151 whole-body cortisol analysis requires a minimum of 0.5g of tissue extract, so since fish were small,
152 we used pooled samples of two or three fish.

153 Tissue cortisol levels were used as an indicator of the stress response. To prevent a possible
154 handling-induced stress response, the time period between capture and killing was < 30 s. Each
155 fish was weighed, minced and placed in a tube containing 3 ml of phosphate buffered saline (PBSg,
156 pH 7.3). The contents were then transferred to a tube containing ethyl ether and subjected to
157 vortexing for then centrifuged after immediately frozen in liquid nitrogen. The thawed portion
158 (cortisol containing diethyl ether) was decanted and transferred to a new tube and evaporated
159 completely, to yield a lipid extract containing cortisol, which was then stored at - 20°C.

160 The tissue extracts were resuspended in PBSg and the whole-body cortisol levels were
161 measured using a commercially available ELISA kit (EIAgen ™ cortisol test,
162 BioChemImmunosystems). This kit is fully validated for zebrafish tissue extracts using the
163 methodology proposed by Sink et al.(2007). The accuracy was tested by calculating the recovery
164 in samples spiked with known amounts of cortisol. The precision was tested by calculating the
165 intra-assay coefficient of variation (CV) of 12 repeated assays in 7 randomly chosen samples on
166 the same plate, and reproducibility was tested by assaying the same samples on different plates
167 and calculating the inter-assay CV.

168 To test for linearity and parallelism, serial dilutions of tissue extracts were performed in
169 the buffer provided with the kit. We detected a strong positive correlation between the curves (R^2
170 = 0.892) and determined that the samples displayed low inter- and intra-assay CVs (7–10 % and
171 5–9 %, respectively).

172 ***2.5. Behavioral quantification***

173 We repeated the methodology employed in Oliveira et al.(2014). Briefly, the water column
174 was divided into three areas of equal size, from the bottom to the surface. The time that fish spent
175 in the bottom area was observed and manually recorded, and the % of the session time for each of
176 these behaviors was calculated later on. The rationale for quantifying fish behavior via observation
177 and manual registration was based on the findings of Speedie&Gerlai(2008), which clearly show
178 that zebrafish responses to alarm substances can be reliably quantified by visual-manual recording
179 as well as through computerized video tracking methods. We quantified the time spent near the
180 tank bottom as an indicator of defensive reactions (Gerlai&Csányi, 1990; Gerlai et al., 2000;
181 Quadros et al., 2016). The duration of this behavior was expressed as a percentage of the total
182 observation session duration. The onset of the time at tank bottom was considered when at least 3
183 out of the 10 zebrafish remain in the bottom area based on previous method and data (Speedie
184 andGerlai, 2008).

185 ***2.6. Statistics***

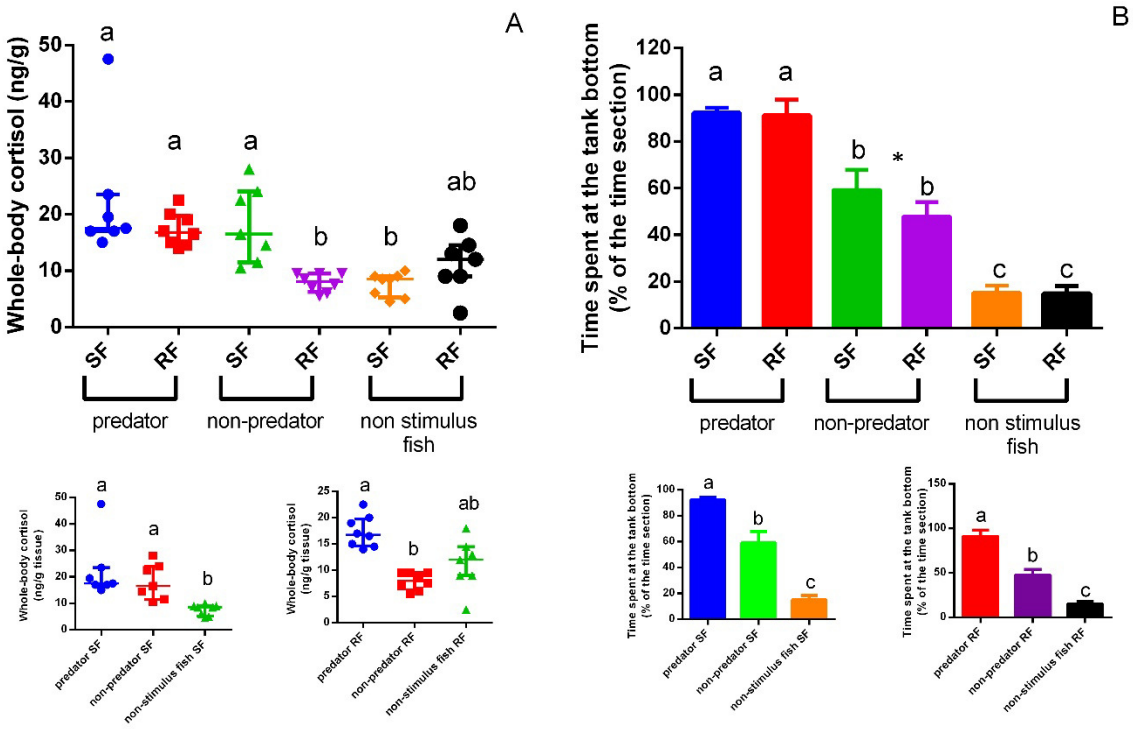
186 For whole-body cortisol and behavior values, we compare all treatments and also
187 proceeded two specific comparisons between zebrafish senders and between receiver zebrafish of
188 experimental conditions. Regarding cortisol values, a Kolmogorov-Smirnov test showed that the
189 samples were derived from populations that did not follow normal distributions. A Bartlett test
190 indicated that the SDs of the samples in the same experiment were statistically indistinguishable.

191 Therefore, we applied the Kruskal-Wallis test followed by Dunn's multiple comparison test.
192 Regarding time spent at the bottom aquaria, the data passed in Kolmogorov-Smirnov test and
193 Bartlett test and, therefore, we applied an one-way ANOVA, followed by Tukey's multiple
194 comparison test to compare the means in each experiment. Significant differences were set at α =
195 0.05.

196 **3. Results**

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

202 Regarding defensive behavior, for fish exposed to the predator, both SF and RF had
203 augmented the time spent close to the tank bottom. In fish exposed to a non-predator fish, this
204 variable was also increased for SF and RF, but this response was less intense (statistically lower)
205 than those SF and RF observed in predator exposure treatment, considering the comparison of both
206 groups (non-predator and predator treatment) with the control group (non-stimulus fish). The
207 aquaria with only water induced no significant change in the defensive behavior (Figure 2B,
208 $P<0.0001$, $F_{5,18}=38.66$, SF comparison $P<0.0001$, $F_{2,9}=50.17$ and RF comparison $P<0.0001$,
209 $F_{5,18}=46.63$). Zebrafish from the two additional controls presented very low cortisol concentrations
210 (2.88 ± 0.74 and 3.63 ± 0.59 ng/g tissue).



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212

213 **Figure 2**. Whole-body cortisol response (A) and time spent in the tank bottom (B) of SF and RF.
214 Data were expressed as median (± interquartile range) in the panel A and as mean (± S.E.M.) in
215 the panel B. Data were compared by Kruskal-Wallis complemented by a Dunn's Multiple
216 Comparisons Test (panel A) and by one-way Anova followed by Tukey´s multiple range test (n =
217 7 – 8 for cortisol and 3 – 4 for behavioral analysis). Different letters above medians or means
218 indicate the statistical difference.

219

220

221 **4. Discussion**

222 We showed that zebrafish visually exposed to predator display antipredator behaviors. that
223 in turn acted as visual alarm cue for conspecific fish. Zebrafish, unexposed to a predator, showed

224 defensive maneuvers when watched their conspecific displaying defensive behaviors. In addition,
225 these visual cues induced activation of hypothalamus-pituitary-interrenal axis (stress response),
226 culminating with a whole-body cortisol increase. These endocrine and behavioral responses might
227 be associated with stress events, like predation risk, since they are well described in the biological
228 interaction between prey and predator in some species (Barton 2002; Clinchy et al., 2011; Archard
229 at al., 2012). Based on our data, we can affirm that eavesdropping occurs via visual sensory system
230 in zebrafish facing their conspecifics in a context of predation risk. Zebrafish in a direct visual
231 contact with a harmless fish, that had an interpretation of a false predation risk, initiated a short-
232 term alteration in their behavior and an irreversible stress response. Here we named an irreversible
233 stress response because we postulated that this cortisol response once triggered shows a typical
234 increase in cortisol levels, that we realized for this group of fish, whereas the behavioral response
235 can be quickly adjusted to the moment. Contrastingly, their conspecifics observing their altered
236 behavior had the capability of interpret and process the information as a non-dangerous situation,
237 evidenced by the lack of alterations in behavioral and endocrine stress responses in receiver fish.
238 Sender and Receiver fish presented elevated cortisol in response to a context where a
239 predator was visually present, hence, a condition of potential imminent predation risk. When in
240 visual contact with a non-predator or with an empty tank, this cortisol increase was not observed
241 in receiver fish. Another interesting result and a response described as an anti-predator behavior
242 (Gerlai, 2003) is the time spent near to the tank bottom, which increased in sender fish when in
243 visual contact whit a predator and also in their respective receiver fish. We can affirm that zebrafish
244 just viewing the behavior of their conspecifics reacting to the predator presence, is able to interpret
245 as a predation imminent risk, and triggers an anti-predatory response. These results indicate a
246 complex form of communication in zebrafish when dealing with predators because further the

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

257 The increased whole-body cortisol in zebrafish when in visual contact with a predator was
258 previously described(Barcellos et al., 2007). Surprisingly, zebrafish with a visual contact with the
259 non-predator fish, presented a similar increase in cortisol levels. We can highlight that the cortisol
260 response for this group (SF-non-predator treatment) was also a response of smaller magnitude
261 compared to zebrafish viewing a predator. Nonetheless, receiver fish viewing these conspecifics
262 had no elevated cortisol. In the non-predator treatment, both sender and receiver fish had an
263 augmented time spent near the tank bottom when compared with the control group (without a a
264 stimulus fish). However, this response was smaller when compared with the predator exposure
265 treatment, being the response of the SF- non predator higher than his respective RF.

266 We affirm that the anti-predator response showed for fish directly visualizing the predator
267 (Sender fish) was a determinant factor for triggering an anti-predatory behavior in receiver ones,
268 confirming that communication of a threat occurred by visual cues in a context of an interpretation
269 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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