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## Chemical communication of handling stress in fish

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### ABSTRACT

We investigated whether juveniles of the nocturnal fish jundiá (*Rhamdia quelen*) and the diurnal fish Nile tilapia (*Oreochromis niloticus*) are able to chemically communicate stress to conspecifics. Groups of 8 fish were reared in tanks under recirculated water (water exchanged among all the tanks) for each species. Fish were handled in half of the tanks (stressor fish) and whole-body cortisol concentrations were compared among handled fish, non-handled fish exposed to water from the handled fish, and non-handled control fish held with no water communication. For each treatment cortisol concentrations were determined before exposure to the stressor (basal levels) and after 1, 2, 4, 8, and 24 h. Basal levels of cortisol confirmed fish were unstressed in the beginning of the experiment. Cortisol was increased in the stressor fish 1 h after handling. Fish receiving water from the stressor fish increased cortisol levels later (2 h after the stressor fish were handled). As the isolated control group maintained cortisol levels unchanged throughout the experiment, we concluded that some chemical factor was released by the stressed fish in the water and thus stressed the conspecifics. This pattern was similar for both unrelated species, thus suggesting that this communication might have evolved earlier in fish and reinforcing the biological value of this kind of information.

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### 1. Introduction

Stressors in fish can be categorized into four types [1]: a) physical factors that directly touch the animal, such as bites [2], high or low temperatures [3], and low oxygen [4]; b) factors released from a stressor that does not involve directly touching the animal, such as chemical or visual cues from a predator [5,6]; c) memory of a stressful condition that is not currently present [1,7]; and d) cues released from a conspecific that was previously stressed [8,9]. Accordingly, while in most of these categories the initial stressor acts on the focus stressed animal, category d is the only to involve social communication of stressor. Here, an example of category d is provided by means of chemical communication. Such putative chemical alarm cues are thought to be produced and stored by epidermal 'club' cells and released into the water as a result of skin injury (i.e., alarm substance) [8,10–14] or by non-injured fish exposed to a stressful condition (i.e., disturbance substance) [9,15].

Disturbance substances would be expected to increase survival by enhancing an individual's awareness of environmental disturbances; however, few studies have documented this phenomenon. On the other hand, physiological stress responses induced by alarm substances have been observed in European minnows (*Phoxinus phoxinus*) [10], pearl dace (*Semotilus margarita*) [11], coho salmon (*Oncorhynchus kisutch*) [12], and Nile tilapia (*Oreochromis niloticus*) [13]. Ide et al. [16] observed behavioral reactions in matrinxã (*Brycon cephalus*) exposed to alarm substances, but failed to detect changes in plasma glucose or cortisol. These authors attributed this failure to the intensity of the stimulus. Toa et al. [17] reported an increase in cortisol but not plasma glucose or hepatic hsp70 in rainbow trout (*Oncorhynchus mykiss*) exposed to a disturbance substance (water) or alarm substance (skin extract) from stressed conspecifics; substances from nonstressed fish did not elicit any response. It is not clear whether stress responses are due to alarm substances [8] or disturbance cues [9]; however, these studies suggest disturbance cues more likely to elicit a stress response, but still further research is needed on this issue.

In the present study, we describe a cortisol response to chemicals released from stressed conspecifics in two fish species: jundiá (*Rhamdia quelen*) and Nile tilapia (*O. niloticus*). Whereas jundiá is a nocturnal silver catfish in which chemical communication is expected, the Nile tilapia is a diurnal fish. The jundiá (Heptapteridae) and Nile tilapia (Cichlidae) are phylogenetically distant, demonstrating the importance of this adaptive response.

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## 2. Material and methods

### 2.1. Animals

We maintained a population of 120-day-old mixed-sex jundiá fingerlings (*R. quelen*) in a 6200-L plastic tank (daily water exchange, 10%) before distributing into experimental tanks. Fish were maintained on a natural photoperiod and fed twice a day (10:00 and 16:00) at 5% of body weight with commercial extruded food (42% crude protein, 3400 kcal kg<sup>-1</sup> digestible energy). Mixed-sex Nile tilapia fingerlings (*O. niloticus*) were 90 days old and maintained under the same conditions as jundiá.

### 2.2. Experimental design

Fish species were evaluated in separate experiments. The strategy, which is schematized in Fig. 1, consisted of maintaining groups of 8 fish in 12 circular tanks (0.25 m<sup>3</sup> each) under recirculated water (water exchanged among all the tanks). Fish were handled in half of the tanks (stressor fish). We then compared whole-body cortisol concentrations among three treatments: handled fish, non-handled fish exposed to water from the handled fish, and non-handled control fish held separately in five non-recirculating tanks. Water supplied, however, was the same for the whole system (experimental and control tanks). Cortisol concentrations were determined before exposure to the stressor (basal levels) and after 1, 2, 4, 8, and 24 h (each group, n = 8 each time point per treatment). As whole cortisol was measured, all fish of a tank in a same time point were killed for cortisol measurement. Thus, each trial measured cortisol in a time point for each treatment, thus avoiding any repeated disturbance effect. All experimental procedures were approved by the Institutional Ethics Committee at the University of Passo Fundo, RS, Brazil.

### 2.3. Procedures

The handling stress consisted of chasing the fish with a net for 60 s, which has been shown to be an effective stressor for both Nile tilapia [18] and jundiá [19]. To prevent management bias, views of adjacent tanks were completely blocked by black covers on all tanks. Moreover, water reached each tank (in and out) from a tap located about 15 cm

above the next tank, thus avoiding any possibility of sound communication between tanks.

During the experiment, fish were fed to satiation by means of a small hole in the black cover. To prevent management stress, feces and food residue were not siphoned from the tanks. Water quality was maintained as follows: temperature, 24.6 ± 0.08 °C; dissolved oxygen, 6.2 ± 0.01 mg L<sup>-1</sup>; pH, 7.3 ± 0.01; and NH<sub>4</sub> and NO<sub>2</sub> never exceeded 0.5 mg L<sup>-1</sup>, total hardness and alkalinity, 60 and 65 mg L<sup>-1</sup> CaCO<sub>3</sub>, respectively.

To determine tissue cortisol concentration, fish were captured and immediately frozen in liquid nitrogen for 10 to 30 s, and then stored at -20 °C until cortisol extraction. To minimize management stress, less than 30 s elapsed between capture and killing. Whole-body cortisol was extracted according to the method of Sink et al. [20] with a minor modification described by Barcellos et al. [18]. Cortisol concentrations were determined from duplicate samples of tissue extract with a commercially available enzyme-linked immunosorbent assay (ELISA) kit, ELAgen™ cortisol test (BioChem ImmunoSystems). This kit was validated for fish tissue extracts [18].

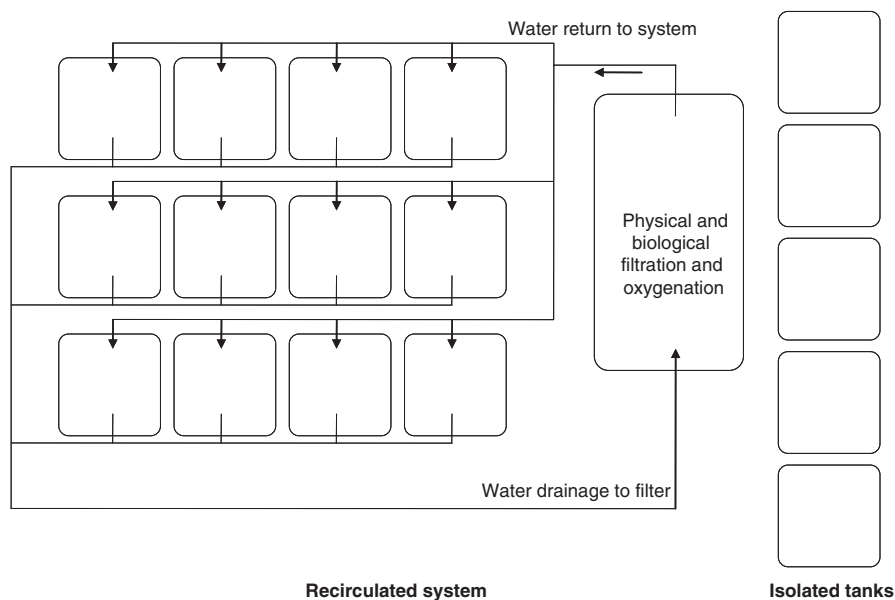
### 2.4. Statistics

The whole-body cortisol values of all treatment groups were compared by two-way analysis of variance (ANOVA), with treatment and time as factors, followed by Tukey's multiple range tests. Statistical significance was accepted at p < 0.05.

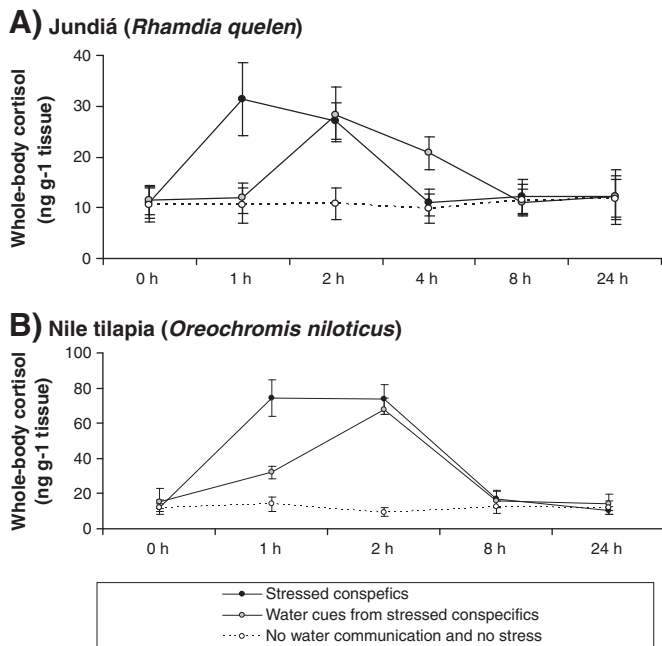
## 3. Results

The 4-h cortisol samples for Nile tilapia failed; therefore, these concentrations were not available for analysis. Basal whole-body cortisol concentrations were similar among treatment groups and remained unchanged in the isolated control groups throughout the experiment (Fig. 2). However, 1 h after handling, cortisol concentrations were increased in the handled fish of both species, and concentrations remained high for at least 1 h. In jundiá, the cortisol concentration of the handled fish was still elevated at 4 h, significantly higher than that of the other groups (Fig. 2A).

Non-handled jundiá exposed to water from stressed conspecifics demonstrated increased cortisol concentrations at 2 h (Fig. 2A). In



**Fig. 1.** Schematic view of the experimental design. Each tank was visually separated from others to avoid interference. Moreover, control tanks were close to the experimental tanks and controlled any external disturbance. Water flow through the tanks was about 2 mL/min, reaching each tank from a height of about 15 cm. The water flows through all the recirculated tanks.



**Fig. 2.** Chemically-mediated increase in whole-body cortisol levels in jundiá (A) and Nile tilapia (B). Exposure to water from handled fish (stressed conspecifics) increased the whole-body cortisol levels in non-handled fish of both species. Note that cortisol levels for Nile tilapia was not measured at 4 h after the stressor and that scales in Y axes are different for A and B. Data are presented as mean  $\pm$  standard error of the mean (each time point,  $n=8$ ).

contrast, cortisol levels of non-handled Nile tilapia were significantly increased as early as 1 h after exposure to water from stressed conspecifics (Fig. 2B). For both species, the area under the cortisol response curve for the non-handled stressor-exposed fish was lower than in that of the handled fish (Fig. 2). Jundiá appeared to produce less cortisol overall due to a slower cortisol increase (Fig. 2A). Because the 4-h cortisol samples for Nile tilapia were not obtained, it is not known whether cortisol concentrations returned to basal levels before the 8-h time point (Fig. 2B).

#### 4. Discussion

Here we report that whole-body cortisol concentrations in fish were increased by chemical cues from stressed conspecifics. This conspecific communication is interpreted as an adaptive mechanism that increases cortisol in anticipation of a potential threat, amplifying the animal's awareness of its environment. Chemical cues overcome visual barriers to communicate stressful conditions to conspecifics in other places. Because this response was similar in two phylogenetically unrelated species, Nile tilapia and jundiá, we suggest that it may be a very ancient mechanism in fish.

The basal whole-body cortisol levels (ng cortisol/g fish) observed in the present study ( $\sim 15$  for Nile tilapia;  $\sim 12$  for Jundiá) were similar with basal ranges reported previously:  $<20$  [Jundiá; 21]; 5 to 15 [zebrafish; 22,23];  $<10$  [minnows; 24];  $\sim 7$  [zebrafish; 25]. Moreover, the similarity between cortisol profiles of the unhandled control fish overtime confirms the reliability of the whole-body cortisol analysis. The strongly elevated cortisol levels in the handled fish further corroborate this method because handling has been shown to strongly increase cortisol in jundiá [18] and Nile tilapia [18,26].

The increased cortisol in the non-handled fish exposed to water from stressed conspecifics indicates that handled fish release a substance that induces a stress response in the recipient fish. This is concluded since the reported cortisol increase cannot be explained by any potential disturbance factor from the experimental system. Water

from stressed fish reached the non-stressed conspecifics by falling from a height of about 15 cm; thus, water could not transfer sound between fish from these tanks. Whole-body cortisol measured at time zero (Fig. 2) also shows that disturbance from water falling did not affect cortisol levels (this hormone was raised only after water contained stressed conspecific's odor entered the system). Moreover, the time lag in peak cortisol concentration between the handled fish and the unhandled fish exposed to recirculated water reinforces that the cortisol increase in these unhandled fish may be a function of water exchange speed and the time required for sufficient quantity of the chemicals to be released by the handled fish.

Another argumentation is that the biofilter used in the present study could not affect any significant water component of the reported cortisol increase. First, the same biofilter was in the tanks of the three treatments. Second, and more importantly, as an effect on cortisol occurred, any explanation in terms of chemicals sequestered or altered by biofilter's microbial activity is meaningless.

Characterization of the putative water-borne substance requires future investigation; however, its effects reveal an important adaptive mechanism. Chemical communication overcomes physical barriers and reaches places that light waves necessary for vision cannot reach. Therefore, chemical cues from conspecific fish may be as important for a diurnal species like the Nile tilapia as they are for a nocturnal species. This kind of chemical communication enables animals to perceive what conspecifics are aware of and react accordingly. For example, pacú (*Piaractus mesopotamicus*), is a diurnal species that can differentiate a predator from a non-predator, retreating and approaching accordingly [9]. Accordingly, pacú watching a predator release chemicals that disperse to conspecifics; when watching a non-predator, they release chemicals that attract conspecifics. This chemically-mediated communication provides additional environmental information to a fish unable to see a potential danger, thus eliciting the appropriate reaction.

In the present study, the cortisol response was similar between Nile tilapia and jundiá. Such chemical communication is expected to require the production of specific chemicals and the expression of specific receptors and cell biochemical machinery to amplify the signal. Despite this complexity, it occurred in two phylogenetically distant species of the Cichlidae and Heptapteridae families. Similar chemical communication is also reported by Mothersill and colleagues for rainbow trout [27], zebrafish [28] and medaka [29] exposed to ionizing radiation stress. Although convergent evolution might have occurred, a more parsimonious explanation assumes that this trait evolved earlier in fish. This explanation reinforces the adaptive value of such a strategy and conservation of a complex mechanism. This strategy may be also part of a wider phenomenon where kairomones trigger beneficial responses in a different species, or even in plants' allelopathic responses.

The present results have practical implications for fish culture. Release of chemical stress factors should be considered when handling fish in a recirculating system. In addition, consequences for catch-and-release practices cannot be ignored, as hooked fish may release chemicals that stress conspecific fish, thereby spreading the damage to many more fish.

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