



## Effects of predator odour on antipredator responses of Nile tilapia



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### HIGHLIGHTS

- Predator odour induce anti-predator behaviour.
- Predator odour induce an increase in ventilation rate.
- Increasing of ventilation rate to predator odour prepare Nile tilapia for 'fight or flight'.
- Predator odour did not activate the hypothalamic-pituitary-interrenal axis.

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### ABSTRACT

Several fish species exhibit antipredator responses when exposed to chemicals which indicate risk of predation. One such substance is the scent of a predator (a kairomone) that may induce defensive responses in a potential prey. In the present study, we show that chemical cues (odour) from predator fish induce antipredator and stress responses in Nile tilapia. When exposed to predator odour, Nile tilapia decreased activity and increased ventilation rate (VR), but no increase in plasma levels of cortisol and glucose was found. Although the hypothalamic-pituitary-interrenal axis (HPI axis) was not activated, an increase in ventilation is a fast response which can provide the fish enough oxygen for a 'fight or flight' event when facing a predator. Thus, this respiratory response suggests an anticipated adjustment in order to prepare the body for a defensive response, such as escaping, irrespective of HPI axis activation.

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### 1. Introduction

In predator-prey interactions, the ability to recognize in advance the threat of a potential predator plays a key role in the survival of prey for any animal species [1]. Several fishes exhibit defensive responses when exposed to chemical cues that indicate predation risk [2]. These cues are well-documented as perceived stressors in fish [2–5] and can evoke a coordinated set of behavioural and physiological responses to cope with the threat, enabling the animal to maintain its homeostasis [5]. These cues can be derived from injured conspecifics, (alarm cues [2,6] conspecific blood [7]), dead conspecifics [8], disturbed and non-injured conspecifics [9], injured heterospecific sympatric species [3] or predator odours (kairomones [10,11]). In the case of predator chemical cues, diet may interfere with detection of predators; for instance, prey may detect a predator's odour more easily, as evidenced by most drastic antipredator responses after a predator has fed on conspecific prey [12,13].

To avoid predation, fish have evolved many strategies to deal with such pressures [14]. In these strategies, prey may elicit behavioural

responses, reducing the risk of detection by decreasing locomotor activity [15] or being immobile [16,17]. Further, to escape from a predator after being detected, prey may migrate [18], flee [19] or seek refuge [20–22]. One common antipredator fish behaviour in response to predator chemical cues is a decrease in swimming activity [6,15,21,23]. Minimizing locomotor activity can reduce water vibrations and detection by predators, increasing prey survival chances [3,15].

Although behavioural responses in fish are generally observed when predator odour is present, physiological responses are also required to overcome the threat of predation [24]. These responses have been broadly described as primary, secondary, or tertiary [5]. Primary responses involve some initial neuroendocrine feedbacks after sympathetic autonomic nervous system activation, resulting in catecholamine release from the chromaffin tissue (hypothalamic–sympathetic–chromaffin axis; HSC) [25], and/or the hypothalamic-pituitary-interrenal (HPI) axis stimulation, culminating in discharge of corticosteroid hormones (e.g. cortisol) into the circulation [26,27]. This is accompanied by rapid cardiorespiratory adjustments [5,28] as VR increases [5,6,29]. Secondary responses occur when stressors related to predation risk persist and are characterized by metabolic changes, such as increased plasma glucose levels [5,26,30]. Additionally, tertiary responses

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may occur in chronic stress situations, constricting the animal's well-being, affecting growth, resistance to disease and reproductive capacity [5,31]. In this context, previous studies have reported antipredator behaviour [9,11,19,20], VR increase [29,32,33], cortisol release [27,34,35] and enhanced metabolic response [36–38] in fish exposed to a predator cue. However, none of these provide a link between primary (antipredator behaviour, and increase of VR and plasma cortisol) and secondary responses (increase of plasma glucose).

We selected the Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758; -Acanthopterygii; Perciformes; Cichlidae) as our experimental model, a cichlid fish species that visually recognizes and executes antipredator behaviour in the presence of predator fish [39,40] and responds to chemical alarm cues [6,41]. Furthermore, this species also exhibits varied responses to different stressors, such as increasing VR, plasma cortisol and glucose levels [6,42,43]. Thus, we performed three experiments to further investigate the hypothesis that Nile tilapia submitted to the odour of a natural predator which had been fed tilapia, the African catfish *Clarias gariepinus* (Burchell, 1822; Ostariophysi; Siluriformes; Clariidae), could detect and recognize these chemical cues as a predation risk and respond accordingly both behavioural and physiologically.

## 2. Material and methods

### 2.1. Fish and stock conditions

Juvenile Nile tilapias were provided by a commercial fish farmer and were kept in three distinct 310 L tanks. Fish were immature, without sex differentiation, with a (mean  $\pm$  SD) standard length of  $6.37 \pm 0.21$  cm and a mass of  $8.60 \pm 1.06$  g. We used as a natural predator the African catfish, kept in a separate 310 L tank, without sex differentiation. As non-predator control, we used the common carp (*Cyprinus carpio*; Linnaeus, 1758; Ostariophysi; Cypriniformes; Cyprinidae), kept in a separate 310-L tank, also without sex differentiation. Predator and non-predator specimens were obtained from a commercial fish farmer. All fishes were maintained in indoor tanks in appropriate stock density (0.5 g/L) for at least 30 days prior to experimentation and fed commercial fish food to satiation once a day (Guabi - Pira 32% protein). Temperature in all tanks was  $26 \pm 1.5$  °C, in which ammonia (<0.5 ppm) and nitrite (<0.05 ppm) levels were controlled by biological filtration and constant water changes (dechlorinated tap water). The photoperiod was kept constant (12L:12D). None of Nile tilapia had previous contact with either African catfish or common carp.

### 2.2. Experimental design

In experiment 1, we tested the effect of predator odour on prey swimming activity. For such purpose, Nile tilapia were exposed to 50 mL of natural predator odour (African catfish) that had been fed tilapia, 50 mL of non-predator odour (common carp, used as control for the predator odour), or 50 mL of water control. Based on similar studies conducted in other fish species that considered changes in swimming activity as an antipredator response [6,15,21,23], locomotion decrease due to African catfish odour indicated that Nile tilapia recognized this chemical as a predation risk, inducing a typical behavioural response. Thereafter, we conducted a second experiment, to investigate the effect of predator odour on VR as an indicator of physiological responses. We exposed Nile tilapia to either 50 mL of predator odour or 50 mL of water control. A rapid VR response to predator odour indicated the Nile tilapia sympathetic autonomic nervous system had been activated and the primary responses (catecholamine and corticosteroid hormones release) had occurred. Results from experiments 1 and 2 provide evidence to hypothesize that predator odour might have stimulated the Nile tilapia HPI axis, thus causing the release of corticosteroid hormones and increased plasma glucose levels. In light of this, we carried out a third experiment in order to evaluate the effect of predator odour on plasma levels of cortisol and glucose over time, in which Nile tilapia were

exposed to 50 mL of predator odour or 50 mL of water control at 0 min, 15 min, 30 min, 60 min, 120 min and 240 min after initial exposure. An increase in cortisol and glucose plasma levels would indicate the HPI axis activation and metabolic responses, respectively.

### 2.3. Predator and non-predator odour collection

Two African Catfish (standard length: 16.20 cm and 16.60 cm; weight: 35.93 g and 43.75 g, respectively) were individually housed for six days in 42 L aquaria ( $40 \times 30 \times 35$  cm) with temperature, photoperiod and water conditions similar to those in the stock. African catfish are active, piscivorous and natural predators of Nile tilapia [44–47] and, to ensure that all predators had been fed and subjected to a standardized procedure, fish were fed twice during this period (second and fourth day) with Nile tilapia fresh fillets (with skin, but scaleless; 3% of African catfish weight per feeding). African catfish necessitate 40 h at a temperature of 30 °C to completely evacuate their gut [48], so on the sixth day (i.e. 48 h after the catfish were last fed, to ensure stomachs were empty), fish were rinsed, transferred and individually housed in 22 L aquaria ( $40 \times 24 \times 23$  cm; stocking density 2 g/L) containing fresh dechlorinated tap water and an air stone, but no filter, and kept for 24 h, without food. After this period, each fish was removed and the water was filtered (to avoid any visible particles), stored into 50 mL aliquots and frozen at  $-20$  °C until required for testing. Non-predator odour was obtained from two common carp (standard length: 15.40 cm and 14.20 cm; weight: 88.43 g and 77.45 g, respectively; stocking density 4 g/L), kept under the same conditions used to collect predator odour. Carp odour was diluted in fresh dechlorinated tap water to attain the catfish odour concentration. Instead of Nile tilapia fillets, carp were fed with the same commercial fish food used in stock conditions (second and fourth day; 3% of carp weight per feeding; Guabi - Pira 32% protein). Procedures were adapted from Ferrari et al. [11].

### 2.4. Experiment 1

We evaluated the effect of predator odour on Nile tilapia behaviour. Fish were exposed to 50 mL of one of the following chemical cues: odour of predator fed on tilapia ( $n = 10$ ); odour of non-predator, used as control for predator odour ( $n = 12$ ); or deionized water ( $n = 9$ ) as a control for presence/absence of chemical cues and lab conditions, totalling 31 individuals. Fish from the stock population were randomly chosen, individually housed and acclimated in glass tanks ( $40 \times 24 \times 23$  cm; 22 L) for 60 h prior to experimentation. Each aquarium was supplied with constant aeration and water temperature was maintained at  $26 \pm 1.5$  °C. Ammonia (<0.5 ppm) and nitrite (<0.05 ppm) levels were controlled by water changes and the photoperiod was kept constant (12 L:12D). Food was offered once a day, but not on the day of the experiment. After the acclimation period, baseline of behaviour was recorded during 5 min and for another 5 min after inserting one of the stimuli into the experimental aquaria. The assessed behavioural response was locomotion, measured by the number of quadrant changes. Each aquarium had on its back a polystyrene plate divided in nine quadrants ( $13.3 \times 8.0$  cm). A quadrant change was considered when at least 75% of the fish's body crossed the line [7]. Locomotion values were considered as the deviation from the initial condition (post-stimulus minus baseline). All movements were quantified using camcorders, set up 1.5 m in front of the experimental aquaria.

### 2.5. Experiment 2

In experiment 1, we found evidence to support the hypothesis that Nile tilapia recognize predator odour as a predation risk by decreasing their swimming activity. Therefore, we conducted a second experiment to investigate whether Nile tilapia VR would increase in response to predator odour. Fish were individually housed and acclimated in the

same setup as described in the previous experiment. We exposed Nile tilapia to 50 mL of odour from a predator fed on tilapia or 50 mL of deionized water. Baseline VR was measured for 5 min before stimuli insertion, and then for another 5 min after stimulus introduction ( $n = 14$  for predator odour;  $n = 16$  for deionized water; 30 fish total). VR was estimated by counting opercular beats (OB) for 15 s and multiplying by 4 to obtain OB per minute (ob/min). Five observations were made over 5 min for each period (baseline and post-stimulus), and then averaged. Values were considered as the deviation from the initial condition (post-stimulus minus baseline).

### 2.6. Experiment 3

Well-established indicators of fish stress responses to predator cues include increase in VR, cortisol and glucose plasma levels. From results obtained in experiments 1 and 2, we hypothesize that predator odour caused primary stress responses by release of corticosteroid hormones, thus leading to increased plasma glucose level. We then tested the effect of predator odour on plasma levels of cortisol and glucose over time. Fish were kept and acclimated in the same experimental aquarium setup as described for experiment 1. Two chemical cues were used here, either odour of a predator fed on tilapia or deionized water. Cortisol and glucose levels were measured at 0 min, 15 min, 30 min, 60 min, 120 min and 240 min after initial exposure ( $n = 10$  independent fish for each stimulus and each interval; 120 fish total). Plasma levels of cortisol and glucose were analysed from blood samples. Each fish was gently removed from the experimental aquaria and placed in a container of benzocaine solution (80 mg/L). After partial anaesthesia [49], blood was sampled by cardiac puncture using heparinized insulin syringes (1 mL) to prevent blood clotting. Blood was centrifuged at 754.65 RCF (g) for 10 min and plasma was collected and frozen at  $-20\text{ }^{\circ}\text{C}$  until required for analysis. Plasma cortisol levels were determined with a commercial enzyme-linked immunosorbent assay ELISA kit (DRG®, International Inc., Cortisol Enzyme Immunoassay Kit) specific for cortisol testing. The Elisa assay has been validated and considered a precise and accurate procedure to assess cortisol in teleost fish [50–52], including Nile tilapia [53]. Plasma glucose levels were assessed by the colorimetric method of Trinder [54].

### 2.7. Data analyses

Data were tested for homoscedasticity by Levene's test and for normality by Shapiro-Wilk's W test. Locomotion values were considered heteroscedastic and not normally distributed even after transformation. We calculated deviation from the initial condition (post-stimulus minus baseline) and the difference in changes between pairs of treatments (three comparisons: predator odour  $\times$  water control; predator odour  $\times$  non-predator odour; water control  $\times$  non-predator) were analysed by separate Mann-Whitney  $U$  test [55]. We used the modified Bonferroni procedure [56] to control the false positive error rate (type 1 error) when multiple significant comparisons are performed. The modified Bonferroni procedure  $[(\alpha * \text{treatments} - 1) / \text{number of comparisons}]$  attests that corrections to the family-wise error rate can be done when the number of comparisons exceeds the number of treatments minus 1 [56]. In experiment 1, we had a total of three treatments in which three comparisons were made; therefore, the family-wise error rate was held to  $p < 0.033$ . Ventilation rate data were also heteroscedastic and not normally distributed, so we also used the Mann-Whitney  $U$  test in which statistical differences were considered significant when  $p < 0.05$ . Plasma levels of cortisol and glucose were normal and homoscedastic, analysed by an independent Student's  $t$ -test at each time point, since we had independent groups. Outlying data were identified and removed for locomotion (5) and VR (2), identified by values outside the range of the mean to which two standard deviations were added or subtracted. Residuals were removed in the top or bottom 2.5% of the distribution [57].

### 2.8. Ethical note

This study complied with the Ethical Principles in Animal Research adopted by the National Council for the Control of Animal Experimentation – Brazil (CONCEA – Conselho Nacional de Controle de Experimentação Animal – Brazil) and was approved by the Ethical Committee for Animal Research from the Instituto de Biociências/UNESP (CEUA – Comissão de Ética no Uso de Animais), protocol 237.

## 3. Results

### 3.1. Behavioural responses

Fig. 1 represents three independent Mann-Whitney  $U$  tests used to compare each possible treatment combination. Presence of non-predator odour did not affect locomotor activity compared to water control (Mann-Whitney  $U$  test,  $Z = -0.213$ ,  $p = 0.862$ ), and no difference was observed between predator and non-predator odour (Fig. 1C; Mann-Whitney  $U$  test,  $Z = 1.483$ ,  $p = 0.149$ ), while presence of odour from predator fed on tilapia fillets caused a decrease in swimming activity compared to water control (Fig. 1D; Mann-Whitney  $U$  test,  $Z = 2.204$ ,  $p = 0.027$ ).

### 3.2. Stress responses

Physiological responses to the chemical cue are presented in Fig. 2. Predator odour increased VR compared to eluent control (Fig. 2A; Mann-Whitney  $U$  test,  $Z = -2.411$ ,  $p = 0.015$ ). Both plasma cortisol (Fig. 2B; Student's  $t$ -test,  $p > 0.05$ ) and glucose (Fig. 2C; Student's  $t$ -test,  $p > 0.05$ ) levels were not affected by predator odour at any time point after exposure to chemical stimuli.

## 4. Discussion

In the present study, we showed that predator odour induces anti-predator behaviour and physiological responses in Nile tilapia. When exposed to predator odour, the model decreases locomotion and increases VR. However, no increase was found in plasma levels of cortisol and glucose. Although the HPI axis was not activated, the respiratory alteration may have anticipated a physiological adjustment to prepare the

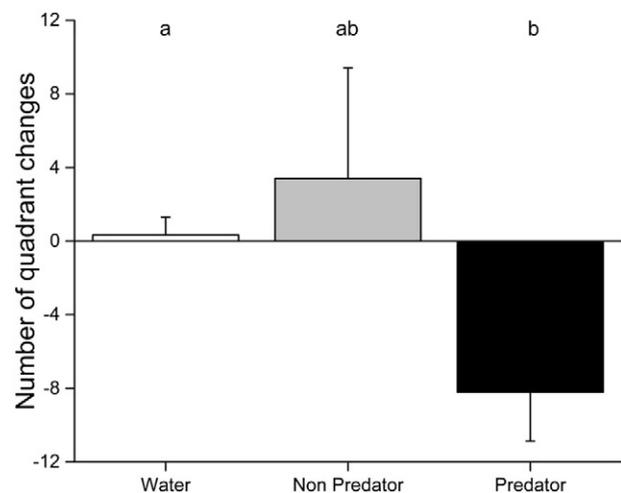
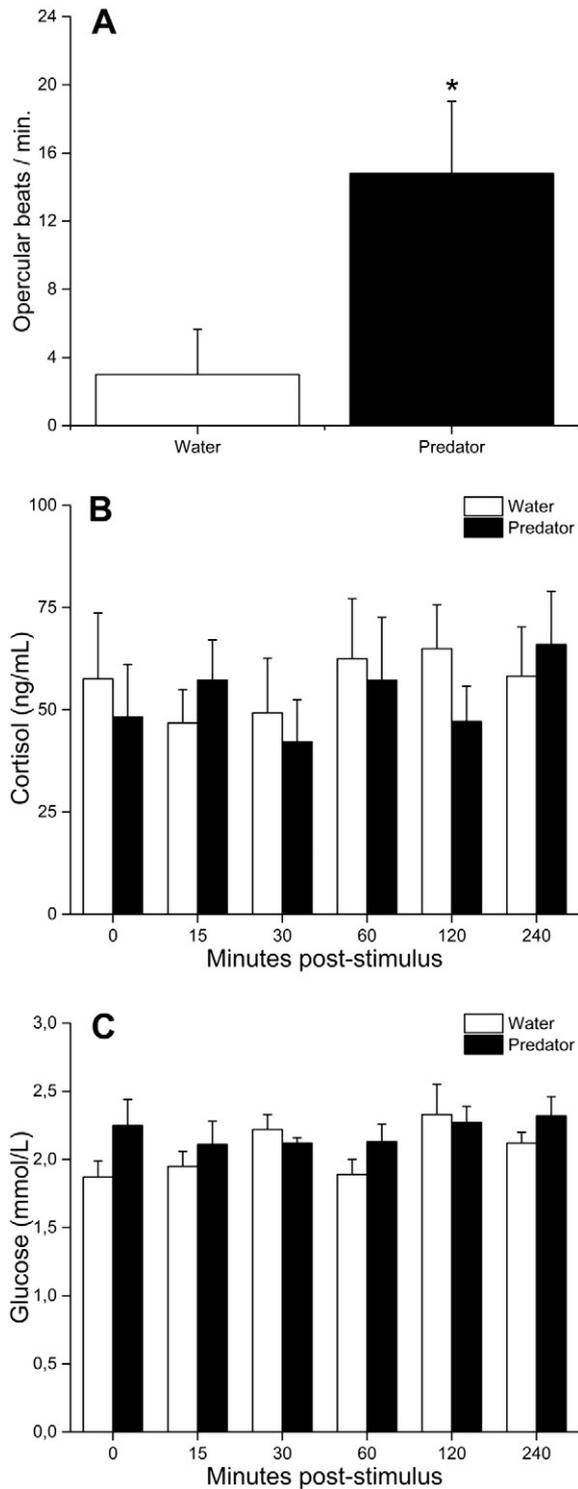


Fig. 1. Behavioural response (locomotion) of Nile tilapia to chemical cues. Number of quadrant changes in fish exposed to non-predator odour compared to deionized water (Mann-Whitney  $U$  test,  $p = 0.862$ ), non-predator odour compared to predator odour (Mann-Whitney  $U$  test,  $p = 0.149$ ) and predator odour compared to deionized water (Mann-Whitney  $U$  test,  $p = 0.027$ ). Locomotion values were considered as the deviation from the initial condition (post-stimulus minus baseline). Data represent mean  $\pm$  SE and different letters indicate significant difference at  $p < 0.033$  (see text for details).



**Fig. 2.** Stress responses of Nile tilapia to chemical cues. Ventilation rate in fish exposed to predator odour and control (A; Mann-Whitney *U* test;  $Z = -2.411$ ,  $p = 0.015$ ); Values were considered as the deviation from the initial condition (post-stimulus minus baseline). Cortisol concentration in blood plasma (B; Student's *t*-test,  $p > 0.05$ ); and glucose concentration in blood plasma (C; Student's *t*-test,  $p > 0.05$ ) in fish exposed to predator odour and control. Data represent mean  $\pm$  SE and asterisk indicates significant difference between treatments at  $p < 0.05$ .

body for a defensive and highly metabolically demanding response, such as escaping or facing the predator [32,58–60].

Some studies have reported antipredator responses in fish prey subjected to the odour of predators fed on prey conspecifics [12,61–63], or even chemical cues from predator faeces [64–67]. In both cases, prey

efficiently recognized the predator's diet and responded more drastically to predation risk when the predator ate prey conspecifics [12,14]. Therefore, these studies showed well-established evidences that prey alarm cues are chemically preserved in the predator, even when passing through the digestive system [68]. African catfish odour donors had their gut emptied before odour collection, suggesting that chemical cues from prey could be derived from urine [69] or mucus [70], for example. Our findings indicate that no active predation events were needed (direct release of prey alarm cues in the water), not even the release of predator faeces or other intestinal by-products as chemical cues, to elicit antipredator responses in Nile tilapia.

Locomotion of Nile tilapia exposed to predator odour was evaluated as an indicator of antipredator response. Swimming activity is strongly affected by chemical cues that indicate predation risk and the reduction of locomotion is considered a defensive response [2,41,71]. In our study, no difference was observed between non-predator and water control, as expected. In contrast, swimming activity was decreased by exposure to chemical stimulus from the predator fed on Nile tilapia, according to other species reports, such as in the fathead minnow *Pimephales promelas*; [12], the brook charr *Salvelinus fontinalis*; [61] and the Atlantic salmon *Salmo salar*; [64]. This decrease in locomotion after recognition of a chemical cue which indicates risk of predation results in lower detection [12,14,22] and predator encounter probabilities [1,10,24,72,73].

After the alteration in behaviour, we evaluated the effects on physiological responses using deionized water as control. Our baseline values of VR and blood plasma concentrations of cortisol and glucose showed an unstressed baseline condition. Herein, fish were approximately 6.4 cm long with a baseline VR of approximately 116 ob/min, very close to non-stressed values found in studies in which Nile tilapia were approximately 6.8 cm long and showed rates of approximately 118 ob/min [74] and 117–124 ob/min [39]. Mean baseline cortisol level was 50.01 ng/mL, in the range of 5.0 and 60.0 ng/mL [42,75–78], previously described as pertaining to a non-stressed situation. Mean baseline glucose level was 2.07 mmol/L, within the range reported by others studies, between 1.91 and 7.21 mmol/L, also demonstrating a non-stressed situation [6,43,79]. All baseline physiological values showed in the present study are consistent with reports mentioned previously and it is important to validate the unstressed baseline condition in which the Nile tilapia started from. The fact that we found no difference in cortisol and glucose levels in Nile tilapia after exposure to predator odour means that the HPI axis was not activated, showing that the only physiological response reported in this study was elevation of VR.

Although the HPI axis was not activated due to exposure to odour of a predator fed on prey conspecifics, Nile tilapia had a decrease in locomotion and an increase in VR when subjected to this chemical cue. Decrease in swimming activity reduces water vibrations that could alert predators, resulting in a lower probability of detection and encounter, thus increasing prey survival odds [2,7,11,21]. Non-activation of the HPI axis makes sense, since in an aquatic environment chemical perception of predators is facilitated [2]. An increase of cortisol, followed by an increase of glucose every time a predator was noticed, could be considered a metabolic fright response [37] and a high-energy demanding process [5,26], leading to a nonlethal energetic cost condition to the prey [37,80,81]. Furthermore, the persistence of a sustained/chronic HPI axis activation could elicit some tertiary stress responses such as reduction of growth rates [24,26,82], reproductive capacity [5,26] and, ultimately, fitness [83].

Surprisingly, increase in plasma catecholamines in response to chemical predator cues in fish has not been described yet. In fish stress condition, catecholamine release might be the major physiological response when an increase in plasma cortisol does not occur [84], since it induces hyperventilation, increases gill diffusing capacity, stimulates blood oxygen transport capacity, and allows a greater uptake in order to support adequate oxygen levels in the blood and tissues [25,84]. Thus, the increase in VR should be enough to augment oxygen uptake and prepare the body for a possible and sudden aerobic activity, like

escaping [32,33,85,86], with minimum but sufficient metabolic adjustment after the first detection of predation risk [37].

This study is an attempt to investigate more deeply the combination of behavioural and physiological reactions of fish in response to chemical stimuli of a predator recently fed on conspecifics. As already shown by Rehnberg and Schreck [30], our results also indicate that a tight linkage between behavioural and prey HPI axis activation due to predator chemical stimuli does not necessarily co-occur. We conclude that Nile tilapia exposed to odour of a predator fed a tilapia diet elicit behavioural and physiological responses by reducing locomotion and increasing VR, but no change in plasma levels of cortisol and glucose occurs. Future studies should take into account analysis of catecholamine levels and examine if activation of the prey HPI axis could happen when predation risk is more intense; e.g. in situations where chemical (predator odour and prey alarm cues) and predator visual stimuli are coupled simultaneously.

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