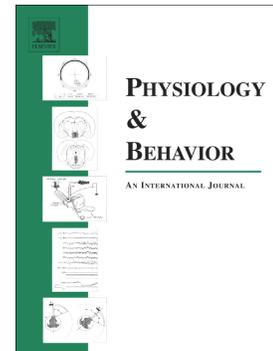


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**Ventilation responses to predator odors and conspecific chemical alarm cues in the frillfin goby**

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**Abstract**

The chemical detection of predation risk is direct when based on predator odors, or indirect when an injured conspecific or heterospecific signal it. Physiological adjustments may be necessary in parallel to defensive reactions to cope with an imminent risk. Here, we tested the effects of predator odors and conspecific chemical alarm cues in ventilation response (VR) of frillfin goby, *Bathygobius soporator*, because this response increases oxygen uptake for supporting behavioral tasks. No VR change was detected in response to odors of predators (catfish) that fed on conspecific, heterospecific fish (tilapia), or were deprived of food and to non-predator (tilapia) that fed chow (non-specific odor control) and odor eluent. The goby's VR, however, increased in response to conspecific alarm cues, but not to heterospecific cues or eluent. Clearly, the VR response in fish depends on the nature of the chemical cue. It is in line with 'threat-sensitive hypothesis' as a chemical cue from an injured prey might mean a foraging predator, whilst the mere presence of a predator odor might not. In addition, because VR can increase, decrease or remains unchanged in response to predation risk in other fish species (including other gobies), we reinforces the species-specific characteristic of VR responses in fish, regarding the results obtained here for frillfin gobies.

Keywords: Alarm substance; Antipredator behavior; Chemical communication, Stress response, Use of public information.

## 1. Introduction

The perception of a potential threat imposed by predators is essential for prey survival. The detection of chemical cues that indicates predation risk can be direct, when threat assessment is based on predator odor (Chivers and Smith, 1998; Kats and Dill, 1998). However, it can also be indirect, when a conspecific or heterospecific prey chemically signals the imminent risk (Chivers and Smith, 1998; Kats and Dill, 1998). In the case of fish, some species have cells located in the epidermis that when damaged by a predator attack release a substance in water (alarm substance) that induces defensive responses in conspecifics (Chivers and Smith, 1998). For instance, detection of alarm substance results in changing the priority of responding to relevant environmental stimuli in fish, such as decreased foraging (Ferrari et al., 2012) or territorial defense (Barreto et al., 2010; Meuthen et al., 2015).

Overall, the behavioral responses in these contexts have been well documented, but the physiological correlates have not. The predation risk (and other stressful situations) increases alertness, and respiratory adjustments can take place. An increase of ventilation rate (VR) might be a way of preparing the body for escaping tasks since a function of the ventilatory system is to supply the body with enough oxygen (Fernandes and Rantin, 1994). In fact, increased VR is a response when fish faces a predation risk based on predator odors (direct cues) in Atlantic salmon *Salmo salar* (Metcalf et al., 1987; Hawkins et al., 2004 a,b, 2007) and Nile tilapia *Oreochromis niloticus* (Miyai et al., 2016) or alarm substances (indirect cues) in Nile tilapia (Barreto et al., 2010; Sanches et al., 2015) and rainbow darters *Etheostoma caeruleum* (Gibson and Mathis, 2006).

Many predators rely on prey's body movements as stimuli to track them (Burrows et al., 1994; Burrows and Gibson, 1995). Thus, responding to a threat by reducing the VR would increase crypsis, particularly if the prey concomitantly adopts a postural immobility to become less susceptible to detection by predators. In addition, if crypsis fails, a decreased VR would not impair an emergency escape maneuver, because a small escape swimming could be performed anaerobically, requiring no elevation of the VR. In this context, in the presence of a foraging

predator, and consequently its odor (direct cue), the strategy of hypoventilation could be a possible action. However, only chemical alarm cues (indirect cues) have been shown to induce hypoventilation in fish, such as in pintado speckled catfish *Pseudoplatystoma coruscans* (Barreto et al., 2012), piauçu fish *Leporinus macrocephalus* (Barbosa-Junior et al., 2012) and the round goby *Neogobius melanostomus* (Pütys et al., 2015).

Fish defensive responses in terms of increase or decrease VR could be supposed as a result of the locomotion strategy adopted: dashing/increased activity or freezing/decreased activity, respectively. However, it is not clear and does not have this positive association between VR and locomotion strategy, because predator odors (Atlantic salmon - Hawkins et al., 2004 a,b, 2007; Nile tilapia - Miyai et al., 2016), as well as alarm substance (Nile tilapia – Barreto et al., 2010; Sanches et al., 2015) can lead to freezing or decreased activity with hyperventilation. Thus, no clear prediction can be made regarding how VR is expected to change with perceived threat.

These disparate observations in VR (hyper- or hypoventilation) point toward the need for better comprehending the effects of chemical cues that indicates predation risk in a greater number of fish species. In this context, we wonder if the cue nature (a direct or indirect indicator of risk) could be an important point. Based on this, the present study evaluates the effects of these direct and indirect chemical cues in ventilation rate of frillfin goby (*Bathygobius soporator* - locally known as amborê). The frillfin goby is an avid predator of rocky shores and estuaries that consume small fish and crustaceans (Lopes and Oliveira-Silva, 1998; Tomida et al., 2012). However, these fish are also predated by larger voracious predators, such as catfish (Chalom et al., 2008). The frillfin goby is also reactive to alarm substance (Barreto et al., 2014). Accordingly, the gobies exposed to this stimulus exhibited sheltering and decreased swimming activity.

## 2. Materials and Methods

### 2.1. Fish and holding conditions

Specimens of Frillfin goby were collected from the estuary of the Itanhaem River in Itanhaem city, São Paulo state, Brazil. Funnel traps baited with shrimp were used for capturing fish. The traps were sunk into the estuary water near rocks. After a few minutes, the traps were withdrawn from the water and the captured frillfin gobies were gently placed into plastic lidded buckets filled with seawater. This procedure was repeated several times to have a suitable sample size. Constant aeration was provided to bucket water, from the field to the laboratory, using battery-operated air pumps and air stones connected via plastic tubing (nontoxic silicone). No mortality was observed. After collection, the fish (mean length of ~ 9 cm, standard length of ~ 7.5 cm and about 6-7 g of body mass) were acclimated in plastic stock tanks for 15 days before experimental procedures (12 fish/70 L water; stock density = 1.0 g/L of water). The tanks were at constant room temperature (~24 °C) using air conditioning equipment. The water was continuously aerated by air pumps connected to the air stones via nontoxic silicone tubing and with biological and mechanical filtrations. The fish were exposed to natural photoperiod and indirect illumination through lab windows. The fish were fed once a day at 4% of biomass with commercial feed for bottom-dwelling carnivorous fish [36% protein; Poytara carnivoros de fundo® (Bottom-dwelling carnivorous fish chow), Poytara LTDA, Araraquara — SP, Brazil].

### 2.2. General experimental strategy

The basic strategy was to evaluate the ventilation rate (VR) of the frillfin gobies, *Bathygobius soporator*, (Valenciennes, 1837; Fig. S1) before and after exposure to chemical cues. These cues used here have the potential to indicate, directly (predator odors - Experiment 1) or indirectly (alarm cues - Experiment 2), a presence of a threat imposed by a foraging predator. All subjects in both experiments 1 and 2 were experimentally naive, used only in a single trial.

In experiment 1 (prey direct assessment of predator presence), the frillfin goby ( $n = 6$  / each condition) was exposed to predator odor (the madamango sea catfish *Cathorops spixii*). Odor was obtained from predators fed on: 1) conspecific (*B. saporator*); 2) allopatric heterospecific (Nile tilapia *Oreochromis niloticus* – to control the specificity of a potential kairomone); or 3) no food (food deprived catfish). In addition, for controlling the specificity of response and lab procedures, response to the odor of: 4) Nile tilapia fed on commercial pellets (nonspecific odor – control kairomone) and 5) artificial seawater without any biological scent (eluent - control) were also assessed.

Experiment 2 (prey indirect assessment of predator presence), we exposed the fish ( $n = 10$  / each condition) to skin extracts of: 1) conspecific (alarm substance); 2) heterospecific fish (Nile tilapia - control alarm substance); and 3) extract eluent (distilled water). This chemical cue of a heterospecific fish was used to confirm if the response of frillfin goby to conspecific skin extract was specific or a general response to chemical stimuli from any injured fish (based on Larson and McCormick, 2005; Manassa et al., 2013). In a previous study (Barreto et al., 2014), frillfin goby was not behaviorally reactive (in terms of sheltering and locomotion activity) to heterospecific alarm substance (skin extract of platy fish *Xiphophorus maculatus*); however, we decided to include a heterospecific control (Nile tilapia skin extract) herein, because we aimed to assess another response variable, the VR, that is a physiological stress variable linked to sympathetic autonomic nervous system. This heterospecific fish, Nile tilapia (Cichlidae), is a phylogenetically distant species from goby species (Gobiidae) and are therefore unlikely to contain the same chemical alarm cues, hence, a suitable heterospecific control fish.

### 2.3. Experimental design

For both experiment 1 and 2 the following experimental protocol was conducted. Specimens of frillfin gobies from the stock population were randomly selected and housed in individual glass

aquaria (23 L; 40 × 23 × 25 cm; water volume of 20 L; 1 fish/aquarium). The fish were acclimated to the experimental aquaria conditions for five days, where they were fed daily as described above, and the leftover food was removed after 1 hour. After this period, fish VR was assessed for 3 min before stimulus presentation (baseline), one time each minute. Next, a chemical stimulus was injected into the experimental aquaria, and fish VR restarted to be assessed 10 s later of that for an additional 3 min (post-stimulus period). The mean baseline and post-cue VRs of each singleton were considered for analysis. A methylene blue dye test demonstrated that the colorant was completely spread throughout the aquarium in ~ 8 s. Behavior recording occurred from 11 h to 14 h in a random order defined by raffle.

#### 2.4. Chemical cues

The predator (catfish) and non-predator (tilapia) odors were obtained following the procedures that we adapted from Ferrari et al. (2006). Odors from catfish that fed on frillfin goby, tilapia or food deprived ones were obtained from six catfish (mean length of ~ 22 cm) individually housed for six days in 23-L aquaria (40 x 23 x 25 cm; water volume of 20 L) with temperature, photoperiod and water conditions similar to the experimental aquarium. Fishes were fed twice during this period (second and fourth day) with prey fillets with skin. On the seventh day, catfish were captured, rinsed with artificial sea water and transferred to other 23-L aquaria, where they were kept for 24 h in the same conditions described before, but were not fed. After this period, each fish was removed and water was filtered into 50 ml aliquots and frozen at - 20 ° C until its use is required for the test trial. The non-predator odor was obtained from two Nile tilapia (mean length of ~ 18 cm, mean weight of ~ 170 g) with the same procedures used to collect predator odor, but Nile tilapia were fed (equivalent of 3% of body mass = 5 g) with commercial fish chow (32% protein). Aliquots of artificial sea water were used as procedures control. The 50-ml aliquots were carefully injected,

with an aid of syringe attached to a nontoxic silicon tubing, onto the surface of the water from behind a curtain.

The skin extract was prepared as described by Giaquinto and Volpato (2001). The skin donor fish were sacrificed by cephalic contusion without the use of anesthetics to prevent interference from chemical odorants. Twenty-five shallow skin incisions were made on both sides of the animal over the entire body. The fish (one tilapia or a goby of similar size - ~ 9 cm) was placed in a beaker with 200 ml of chilled distilled water (extract eluent) and carefully swirled for 5 min. The mixture was filtered through 185 mm filter paper (40 Whatman) to remove any remaining particles. The remaining solution was kept on ice during the experimental procedures. A 10-ml volume of the filtrate was applied to the tank containing the test fish using a syringe [a filtrate volume based on previous observations by Barreto et al. (2014)].

### *2.5. Ventilation rate quantification*

The VR of frillfin gobies was measured by direct observation (Gibson and Mathis, 2006) and was quantified by measuring the time needed for 20 successive opercular or buccal movements to occur (Alvarenga and Volpato, 1995). VR was expressed as opercular beats per minute.

### *2.6. Experimental aquaria conditions*

The experimental aquaria were equipped with air stones, each of which was connected to an air pump by nontoxic silicone tubing for constant aeration. Artificial sea water was used to avoid any environmental scent that might be present in natural seawater. The water temperature was ~ 24 °C, the pH was ~ 8.0, specific gravity ~ 1024 and salinity ~ 33.5 ppt. The concentrations of nitrite and

ammonia were lower than 0.5 and 0.01 mg/l, respectively. The photoperiod and illumination were natural.

### 2.7. Data analyses

The data's normality and homoscedasticity were assessed by using the Shapiro–Wilk W test and Levene's test, respectively. The data satisfied these premises for the use of parametric tests. The data of experiments 1 and 2 were analyzed using a 2-way mixed ANOVA [a between-subjects categorical predictor (chemical cues) and a within-subject repeated measures (time – before and after cue presentation) ANOVA], followed by a Duncan test when necessary. The VR was the dependent variable in these models. The statistical results were considered significant when  $p < 0.05$ .

## 3. Results

In experiment 1, goby VR was assessed before and after exposure to predator odors (Fig. 1). No effects of the chemical cue ( $F_{(4;25)} = 0.95$ ;  $p = 0.45$ ), time ( $F_{(1;25)} = 0.12$ ;  $p = 0.73$ ) or interaction between time and chemical cue ( $F_{(4;25)} = 0.54$ ;  $p = 0.70$ ) were found. However, in experiment 2, wherein fish VR were quantified before and after exposure to conspecific skin extract, ANOVA revealed a significant interaction between time and chemical cue ( $F_{(2;27)} = 21.18$ ;  $p = 0.000003$ ; Fig. 2), indicating that chemical cue and time conjointly induced an effect in VR (isolated effects of the categorical factors were: chemical cue,  $F_{(2;27)} = 2.55$ ;  $p = 0.097$ ; and time  $F_{(1;27)} = 15.06$ ;  $p = 0.0006$ ). The found interaction indicates that conspecific skin extract induced a significant increase in frillfin goby's VR compared to baseline levels. This effect was not observed for goby exposed to heterospecific skin extract or extract eluent. Moreover, post-stimulus VR of gobies were

significantly higher for fish exposed to conspecific cue than VR of those fish exposed to heterospecific cues or extract eluent.

The ANOVA also showed that, before stimuli presentation, in both experiment 1 and 2, the mean basal VRs were statistically indistinguishable among groups. This analysis indicates that fish started from similar resting VR.

#### 4. Discussion

In this study, physiological responses of frillfin goby to threatening chemical cues are dependent on cue type. Herein, goby exposed to conspecific chemical alarm cue (indirect cue) increased the VR, whereas predator odor (direct cue) induced no change in VR. Thus, our data indicates that the nature of the cue (direct or indirect cues) is a factor influencing the VR response profile. Reinforcing this possibility, zebrafish (*Danio rerio*) retreated or were non-reactive in response to water conditioned with cues emanating from stressed conspecifics exposed to different types of stressors (Abreu et al., 2016). Moreover, considering that the round goby (*N. melanostomus* - Pütys et al., 2015), therefore a phylogenetically close fish to frillfin goby, showed a decreased VR in response to conspecific alarm cues, we suggest a species-specific quality of VR response when fish are perceiving a potential predator threat.

In aquatic environments, information about predation risk often occurs via chemical signals (Wiseden and Chivers, 2006). Predator-prey interactions produce chemicals information indicating the proximity and magnitude of a predation threat and this information can result in different behavioral defensive responses (Chivers and Smith, 1998). For instance, the chemical cues of the conspecific skin extract induce a change in prey fish priority to respond to relevant factors (stimulus filtering), decreased foraging (Foam et al., 2005) or territorial defense (Barreto et al., 2010). Here, the defensive strategy regarding respiratory adjustment of the frillfin goby consisted of evaluating

the cue from an injured conspecific and display a stress response (increased VR). This physiological regulation increases oxygen uptake, supplying the body with enough oxygen for any behavioral task performed aerobically (Fernandes and Rantin, 1994). The presence of an injured conspecific prey clearly indicates a nearby presence of a predator that is highly motivated to eat, hence, a risky situation. Public chemical information of predation risk can be provided by neighbors and induce defensive reactions (Wisenden and Chivers, 2006). This strategy of increase VR in frillfin goby induced by chemical alarm cue obtained via publicly available information is plausible in this species because they live in close proximity to conspecifics (Mendes, 2006). Thus, the individual that detect and respond to the chemical cues of the conspecific have more chance of survival.

Prey can detect the threat by a predator odor (kairomone) or odor associated with the prey in the digestive tract of the predator (dietary cues). However, in this study, no difference in VR after exposure to the predator odor fed with conspecific or heterospecific compared to basal levels of VR took place. It is known that prey can get risk information evaluating the concentration of chemical signal in water (Chivers and Smith, 1998). High concentrations may indicate an imminent risk of predation (Chivers and Smith, 1998; Ferrari et al., 2009). Thus, it is plausible to think that skin extract is a more concentrated chemical signal, indicating more readily that there may be a predator motivated to eat and to increase VR enable the necessary adjustments to the animal in the case of an emergency defensive reaction. Moreover, in this context, the absence of response to the presence of catfish odor is consistent with the predictions of the threat-sensitive hypothesis. The threat-sensitivity hypothesis predicts that prey animals alter their intensity of predator defensive responses according to the magnitude of the threat, representing, therefore, an adaptive advantage for avoiding unnecessary time and energy loss (Helfman, 1989). To respond to the mere presence of a catfish odor could lead to the execution of many unnecessary defensive actions, a condition that would be highly energy expensive for the gobies. Hence, prey might decrease their level of antipredator behavior and redirect their efforts to other behaviors that also contribute to increasing survival (*e.g.* foraging and other resources acquisition – Roux et al., 2014).

The presence of predator odor may indicate the presence of predator, but not necessarily an imminent risk, because it could not be a foraging predator, therefore, it could explain the absence of VR changes in response to predator odors. However, to increase VR due to the presence of chemical cues of injured conspecific indicate a more immediate risk, because suggest the presence of a foraging predator (e.g. one that struck and injured a conspecific goby). Since prey responses to predators have costs (e.g. metabolic costs - Cooke et al., 2003), unnecessary and constant defensive reactions may represent high energy expenditure and time consumed in an needless activity in the case of frequent responses to non-foraging predators. At same time, a presence of a non-foraging predator is not a threat until it initiate foraging activities. Thus, considering the absence of response to predator odors, a possibility may be related to frillfin gobies to live in dense assemblages (groups from 1 to 40 fish into an area of 4 m<sup>2</sup> – Mendes, 2006), characterizing a potential dilution of predation risk. In fact, a dilution of predation risk is effective when prey are exposed to a foraging predator because for any one predator attack, the larger the group of prey animals, the smaller the chance that any particular individual will be the victim (Foster and Treherne, 1981). Moreover, frillfin goby changes body color (e.g. from pale to dark body) according to background (Tavolga, 1950) and, frequently, stay inside shelters (e.g. rock crevices), that represents some ways of being safe from predator attacks. Thus, to avoid high costs of frequent unnecessary defensive responses due to the mere presence of a predator, defensive reactions occur when there is a more clear indicator of foraging predator (alarm chemical cue released by injured conspecific) and other factor might improve safety against predator in frillfin goby (e.g. dilution effect, sheltering and camouflage) when a foraging predator is not clearly detectable.

Alternatively to the above statements, the chemically mediated perception of risk by prey fish was recently shown to vary as a function of the amount of prey biomass consumed by a predator (Weissburg and Beauvais, 2015). Thus, the absence of response to predator odors herein could be explained by a less concentrated scent, although we followed a successful protocol to produce a kairomone (Ferrari et al., 2006). However, it must be clarified in future studies, especially because

prey can be unreactive to predator odor and may suffer no influence of predator diet (Scherer and Smeets, 2016) and diet-derived cues could be specific enough and would result in lots of spurious reactions (Weissburg et al., 2014).

ACCEPTED MANUSCRIPT

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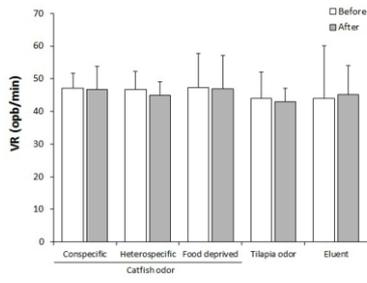
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#### Figure Captions

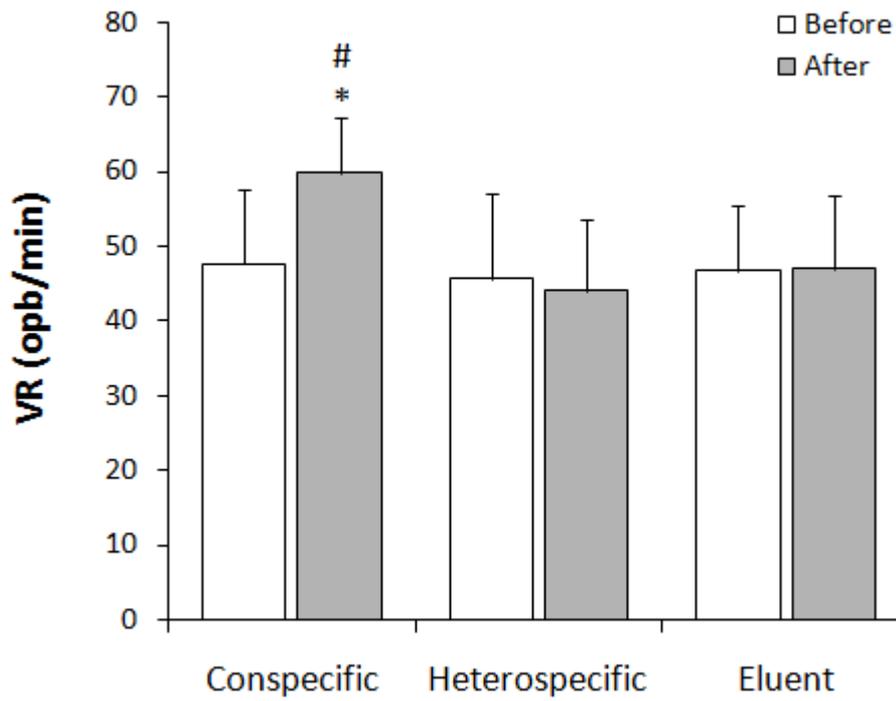
Figure S1. *Bathygobius soporator* (Valenciennes, 1837).

Figure 1. Ventilation rate of frillfin goby *Bathygobius soporator* exposed to predator odors. Mean values ( $\pm$  SD; N = 6 fish each condition). ANOVA showed no effects of the chemical cue ( $F_{(4;25)} = 0.95$ ;  $p = 0.45$ ), time ( $F_{(1;25)} = 0.12$ ;  $p = 0.73$ ) or interaction between time and chemical cue ( $F_{(4;25)} = 0.54$ ;  $p = 0.70$ ).

Figure 2. Ventilation rate (VR) of frillfin goby *Bathygobius soporator* exposed to skin extracts. Mean values ( $\pm$  SD; N = 10 fish each condition). ANOVA revealed a significant interaction between time and chemical cues ( $F_{(2;27)} = 21.18$ ;  $p = 0.000003$ ). \* indicates a significant increase in frillfin goby's VR compared to baseline levels for conspecific chemical cues condition. # indicates that post-stimulus VR of fish exposed to conspecific chemical alarm cue is higher than the other post-stimulus VRs. Baseline values of VR were statistically indistinguishable. .



ACCEPTED MANUSCRIPT



#### Highlights

- Conspecific chemical alarm cues increase ventilation rate in frillfin goby
- Predator odor had no effect on goby ventilation rate
- Predator diet did not influence goby detection of predator odor