Conditional approach as cooperation in predator inspection: a role for serotonin?

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Abstract

In guppies (*Poecilia reticulata*), a small number of individuals break away from a shoal and approach a potential predator, a behavior termed “predator inspection”. These animals employ a “conditional approach” strategy, in which an individual proceeds towards the predator in the first move and subsequently reaches it, but solely if a second individual swims nearby during the entire process (including inspection). This strategy is analogous to the “tit-for-tat” scenario of the Prisoner’s Dilemma, suggesting that is could be used to study cooperative trade offs. Exposure to the animated image of a predator in a tank that contained a parallel mirror – mimicking a reciprocating conspecific – promoted inspection, but increase freezing, suggesting that conditional approach is also associated with fear. To understand whether serotonin participates in conditional approach in guppies, we treated animals with either vehicle (Cortland’s salt solution), fluoxetine (a serotonin transporter inhibitor) or metergoline (a non-selective serotonin receptor antagonist), and tested then in a predator inspection paradigm. Fluoxetine increased predator inspection, while metergoline had no effect. Fluoxetine also decreased time spent avoiding the predator however, it also increased freezing, similarly to metergoline. These results suggest that phasic increases of serotonin levels promote conditional approach, indicating a role of this neurotransmitter in cooperation.

Keywords: Serotonin; Cooperation; Conditional approach; Predator inspection; Guppy

Introduction

Sometimes being seen is better than mingling into the crowd. And when it comes to interactions between putative preys and their predators, it may not be enough to stay vigilant as to simply detect a predator more rapidly, but one also needs to signal its presence and awareness (e.g. head bobbing and fin flicking; (Brown, Godin, & Pedersen, 1999)) and sometimes even to provide information regarding own physical condition (e.g. the leaping in antelopes; (Caro & Allen, 2017)). Indeed, predators seem to be less successful when prey are fully aware of their presence, in part due to the improved vigilance effect in which groups are able to detect predators sooner than solitary individuals (Caro, 2005). One seemingly paradoxical behaviour occurs when some of these preys leave the shoal to slowly approach and inspect the predator presumably to reduce the uncertainty of the situation and to be able to gain valuable information to all other conspecifics involved (Dugatkin, 2013). Since this behaviour implies costs to the individual(s) that interacts with the predator but contributes to the fitness of the shoal, it has been interpreted as cooperative, however, not without strategic specificities.

Conditional approach is a behavioural strategy used by different fish species, including guppies *Poecilia reticulata* (Dugatkin, 1988, 1991, 1992) and sticklebacks *Gasterosteus aculeatus* (Huntingford, Lazarus, Barrie, & Webb, 1994; Milinski, 1987), and acts as an incentive for predator inspection, given that the risk of approaching a predator is shared between all inspectors, but not with the individuals which keep a distance (Bshary & Oliveira, 2015; Dugatkin, 1997). Conditional approach represents a cooperative strategy, similar to tit-for-tat in the Iterated Prisoner's Dilemma (Axelrod &

Hamilton, 1981), allowing the advantages of inspecting the predator to overcome those of remaining at a safe distance (Dugatkin, 1997). Trivers (Trivers, 1971) proposed the Prisoner's Dilemma as a mechanism to interpret the evolution of reciprocal altruism, and Axelrod and Hamilton (Axelrod & Hamilton, 1981) showed that tit-for-tat is an evolutionary stable strategy. This strategy instructs the player to cooperate in the first move and to copy subsequent moves by the partner. Prisoner's Dilemma therefore models a situation in which each individual receives a worse pay-off for not cooperating than for cooperating (Axelrod & Hamilton, 1981; Bshary & Oliveira, 2015; Trivers, 1971). In conditional approach, non-cooperating individuals also obtain information regarding the predator without getting in risk, but mutual reciprocal cooperation offers animals a better pay-off.

The guppy (*Poecilia reticulata*) is a small ovoviviparous fish originating from Central American and northern South America (Magurran, 2005). It is well known by its bright colours, and widely used in ornamental aquaria. Seghers (Seghers, 1973) observed that some guppies, when facing a predator at a distance in the wild, soon inspect this potential threat. Guppies leave the shoal individually or in small groups, and approach the potential threat to obtain information. There is evidence that guppies use conditional approach during this inspection behaviour (Dugatkin, 1988, 1991, 1992, Dugatkin & Alfieri, 1991, 1992). Dugatkin (Dugatkin, 1988) suggested that conditional approach presents the basic characteristics of tit-for-tat: it is "nice" (that is, the first "move" is to cooperate by initiating inspection), "retaliatory" (animals defect by retreating from inspection if the conspecific does not reciprocate), and "forgiving" (animals cooperate by re-initiating inspection if a previously defecting conspecific changes its course and

begins inspection).

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine that is synthesized from tryptophan. This monoamine participates in the modulation of stress and defensive behaviour in fish (Herculano & Maximino, 2014; Soares, Gerlai, & Maximino, 2018; Winberg & Thörnqvist, 2016), and has been implicated in vertebrate social behaviour (Insel & Winslow, 1998; Soares, Gerlai, et al., 2018). Converging evidence from human and non-human animal research suggest that altering 5-HT levels can directly influence social perception and mood, with decreased serotonin leading to isolation and decreased sociality (Kiser, SteemerS, Branchi, & Homberg, 2012). Moreover, it has been shown that 5-HT participates directly in pro-social and cooperative behaviour: tryptophan depletion reduces cooperation in the Prisoner's Dilemma in human participants, and decreases the probability of cooperative responding given previous mutually cooperative behaviour (Wood, Rilling, Sanfey, Bhagwagar, & Rogers, 2006).

In cleanerfish *Labroides dimidiatus*, acute treatment with fluoxetine or the 5-HT_{1A} receptor agonist 8-OH-DPAT increased client inspection and tactile stimulation, while treatment with the tryptophan hydroxylase inhibitor *para*-chlorophenylalanine or the 5-HT_{1A} receptor antagonist WAY 100,635 decreased client inspection and cleaners' cheating levels (Paula, Messias, Grutter, Bshary, & Soares, 2015). Moreover, WAY 100,635 also delays learning of client value, suggesting that this receptor is involved in the perception of danger and therefore in how cleaners appraise, acquire information and respond to client-derived stimuli (Soares, Paula, & Bshary, 2016).

Given that predator inspection entails the evaluation of the level of predator threat, as well as of the value of cooperating vs. defecting with conspecifics, it is probable that 5-HT is involved in the mediation of conditional approach/cooperative behaviour in guppies. We modified the method described by Dugatkin (Dugatkin, 1988), using a video animation of a sympatric predator (*Aequidens pulcher*) presented to a single guppy in a tank with a parallel mirror. We predicted that the predator animation, compounded with the parallel mirror – mimicking a conspecific perfectly reciprocating approach and retreat –, would elicit predator approach (inspection), leading animals to spend more time in the inspection section than near the predator rather than in the avoidance zone, or in the refuge. We also predicted that the predator animation would elicit fear-like behaviour, including freezing and erratic swimming, as well as higher time spent in the avoidance zone and in the refuge. The first prediction was confirmed, but the second prediction was only partially true, given that the animated image increased freezing, but not time in the avoidance or refuge zones. We also tested the hypothesis that serotonin promotes conditional approach by treating animals with fluoxetine or metergoline, a non-selective serotonin receptor antagonist. We predicted that phasic 5-HT (increased by acute fluoxetine) would promote conditional approach, while simultaneously increasing fear/anxiety (as observed in zebrafish: Maximino et al., 2013); blocking receptors with metergoline (thereby decreasing the role of tonic 5-HT) would decrease conditional approach and decrease fear. The first prediction was confirmed, but metergoline only decreased freezing, without affecting conditional approach.

Methods

Animals, housing, and baseline characteristics

For the experiments of the present research, 46 male guppies were used. Animals were bought from a commercial vendor, and left to acclimatize to the laboratory (LaNeC) for two weeks before beginning experiments. Animals were fed daily with commercial feed. The animals were housed collectively in 40 L tanks, at a maximum f 25 fish per tank, with the following water quality parameters being controlled: dissolved oxygen ≈7.8 mg / L, ammonia <0.002 mg / L pH 7.0 - 8.0, temperature 24-30 °C.

Induction of conditional approach

A glass tank (40.6 cm x 18 cm x 25 cm) was used, containing an artificial plant in one extreme ("refuge zone"), and a computer screen (Samsung T20c310lb, 20", LED screen, brightness 20 cd/m^3), positioned on the opposite extreme. A mirror was positioned in parallel to one of the sides of the tank for the whole duration of experiments. The avoidance zone is defined as the half of the tank that is farthest from the screen, and the inspection zone is defined as the other half of the tank (Figure 1)**.** Conditional approach was induced by playback of an animated predator (*Aequidens pulcher*), produced and presented using LibreOffice Impress (Version: 5.3.7.2) on a CPU running Linux 4.15 (Xubuntu 16.04).The animation was presented at the computer

screen for the last 6 min of each session, with the exception of the “Predator absent” condition of Experiment 1.

Open science practices

The experiments were not formally pre-registered. Datasets and scripts are available from https://github.com/lanec-unifesspa/TFT (doi: 10.5281/zenodo.1290044).

Experiment 1

Experimental design, endpoints, and statistical analysis

In the first experiment, 16 animals were used, divided equally and randomly between two groups: “Predator absent” and “Predator present”. For both groups, the mirror was positioned in parallel to the side of the tank. In the first group, animals were introduced to the tank and allowed to explore it for 10 min; in the 5th minute, the video was not turned on. The second group was subjected to the same manipulations, except that the video was turned on in the 5th minute. The order of subjects was randomized using http://randomization.com/. Video recordings of the behaviour were made and later analyzed with manual event recording, with the help of the software X-Plo-Rat 2005 (https://github.com/lanec-unifesspa/x-plo-rat; Tejada, Correio, & Morato, 2018) by two trained observers. Observers were not blind to group allocation, as it was not possible to exclude the portion of the video that included the monitor. Times spent in the avoidance, refuge, or inspection zone were computed as percent change, as follows:

These values were compared using unpaired samples T-tests, with *p*-values < 0.05 considered statistically significant. Effect sizes for these variables were reported as Cohen's *d*. Data on freezing was analyzed as raw values, in s, of the time spent freezing. These data were analyzed using a repeated measures ANOVA, with time block as within-subjects effect, and group as between-subjects effect. Effect sizes for freezing were reported as ω^2.

Experiment 2

<u>Drug treatments</u>

Fluoxetine hydrochloride (CAS #54910-89-3) was bought from Libbs. Metergoline (CAS #17692-51-2) was bought from Virbac Brasil. All treatments were acute – i.e., a single injection was made, 20 min. before experiments. The fluoxetine dose was based on previous work with zebrafish (Maximino et al., 2013), which showed an antipanic-like effect of this dose in an acute treatment. The metergoline dose was based on previous work with rats (Gigliucci, Niamh, Nunan, O'Shea, & Harkin, 2010), which showed an effect of this dose on the forced swimming test.

Injection procedure was adapted from a protocol existing for intraperitoneal administration in zebrafish ze(Kinkel, Eames, Philipson, & Prince, 2010). Briefly, animals were anesthetized in cold water (12 ºC - 17 ºC), and transferred to a "surgical bed" made of a washing sponge, in which a through was cut. The sponge was soaked with ice-cold water to maintain gill perfusion, and the setup was kept on ice. Drugs were injected at a volume of 2 µL/animal, using a Hamilton© microsyringe (701N, needle

gauge 26s), and animals were immediately transferred to aerated water at room temperature to recover.

Experimental design and statistical analysis

In the second experiment, 30 animals were used, divided equally and randomly between three groups: a control group (CTRL), injected with vehicle (Cortland's salt solution; https://wiki.zfin.org/display/prot/Cortland%27s+salt+solution); a fluoxetine-treated group (FLX), injected with 2.5 mg/kg fluoxetine; and a metergoline-treated group (MET), injected with 1 mg/kg metergoline. Experimenters were blinded to treatment by using coded vials for drugs. 20 min after injection, animals were individually transferred to the test tank, and subjected to the same manipulations as the EXP group from Experiment 1. The order of subjects was randomized using http://randomization.com/. Data were recorded and registered as described in Experiment 1, with spatiotemporal endpoints (time spent in avoidance, refuge, and inspection zones) indexed as percent change, and data for freezing analyzed raw. Spatiotemporal data was analyzed using one-way ANOVAs, and freeing data was analyzed using a repeated measures ANOVA, with time block as within-subjects effect, and treatment as between-subjects effect. Effect sizes for freezing were reported as ω^2.

Results

Experiment 1

The animated predator increased time in the inspection zone when the mirror was parallel to the tank (Figure 2A; $t_{[df = 7.3542]} = -2.505$, $p = 0.03912$, $d = -1.25$). The animated predator did not change time in the avoidance zone (Figure 2B; $t_{[df = 7.082]} = -0.9836$, $p = 0.3577$, $d = -0.49$). Refuge use was not altered by the animated predator (Figure 2C; $t_{[df = 7.2327]} = 1.6326$, $p = 0.1452$, $d = 0.82$). Finally, the animated predator increased freezing in the second time block (i.e., after the stimulus was turned on), but not in the first time block (Figure 2D; $F_{[1, 28]} = 6.46$, $p = 0.0169$, $\omega^2 = 0.138$ for the main effect of group).

Experiment 2

Acute fluoxetine increased time in the inspection zone ($p = 0.006$ vs. control), but metergoline had no effect ($p = 0.147$ vs. control)(Figure 3A; $F_{[2, 27]} = 14.62$, $p = 4.99 \cdot 10^{-5}$, $\omega^2 = 0.476$. A similar effect was observed in time in the avoidance zone (Figure 3B; $F_{[2, 27]} = 19.68$, $p = 5.34 \cdot 10^{-6}$, $\omega^2 = -0.555$), with fluoxetine decreasing time in the avoidance zone ($p = 0.001$ vs. control), and metergoline having no effect ($p = 0.095$ vs. control). Neither fluoxetine nor metergoline altered refuge use (Figure 3C; $F_{[2, 27]} = 0.95$, $p = 0.399$, $\omega^2 = -0.00331$). Freezing was increased by fluoxetine ($p =$

$4.0 \cdot 10^{-7}$ vs. control), while metergoline decreased it ($p < 4.0 \cdot 10^{-7}$ vs. control)(Figure 3D; $F_{[2, 27]} = 26$, $p = 5.2 \cdot 10^{-7}$, $\omega^2 = 0.255$ for the interaction term).

Discussion

In the present work, we replicated the observation made by Dugatkin (1988, 1991) on conditional approach by guppies during predator inspection, and extended his findings by showing that guppies also freeze more during predator inspection. We also show that phasic serotonin promotes conditional approach/cooperation but increases freezing/fear, while no evidence was found for a tonic participation of serotonin in conditional approach.

We predicted that the animated predator image would promote predator inspection, but would also increase fear, reflected by increased freezing (Brown & Godin, 1999), as well as time spent in the avoidance and refuge zones. While freezing did increase, animals did not appear to avoid the predator or try to keep a safe distance (e.g. flee). We suggest that freezing, in this situation, is an optimal strategy employed by the animals to avoid being detected by the predator while at the same time, it can benefit from gathering information, by maintaining a higher level of alertness. The relationship between fear, stress, and social behaviour has seldomly been addressed in the literature (Soares, Cardoso, Carvalho, & Maximino, 2018; Soares, Gerlai, et al., 2018). There is some evidence from caribbean cleaning gobies *Elacatinus* spp. that in cleaning mutualisms, inspecting piscivorous clients (potential predators) is stressful, as cortisol

levels rise during these interactions (Soares, Bshary, Cardoso, Côté, & Oliveira, 2012). These cortisol changes enable for greater levels of attention, making cleaners to immediately respond to these dangerous clients (approaching and inspecting) while securing their own life and of all remaining potential clients nearby (Soares et al 2007). Interestingly, field experiments aiming at the cleaner wrasse *L. dimidiatus* showed that treatment with cortisol increased their deceptive tendencies, with cleaners providing more tactile stimulation to smaller clients with their pectoral and pelvic fins – a behaviour that attracts larger clients that are then bitten to obtain mucus (Bshary 2002, Soares, Cardoso, Grutter, Oliveira, & Bshary, 2014). On the other hand, blocking glucocorticoid receptors led cleaners to increase tactile stimulation to larger clients (Soares, Cardoso, Grutter, Oliveira, & Bshary, 2014), which are the normal recipients of greater amounts of physical contact (Bshary & Wurth, 2001), similarly to predators (Grutter, 2004).

In Experiment 2, we hypothesized tonic and phasic roles for serotonin in promoting cooperation and increasing fear. We found that acute fluoxetine increased inspection and also fear, while acute metergoline only decreased fear. Guppies under treatment with fluoxetine spent more time in the inspection zone (i.e. nearer to the image of a predator) and less in the avoidance zone. However, fluoxetine also increased freezing, suggesting that its effects were not fully explained by simply increasing “boldness” (or, conversely, decreasing fear and/or anxiety). In the cleaner wrasse *L. dimidiatus*, fluoxetine increased the propensity to inspect clients without altering cleaning quality (Paula et al., 2015), suggesting that phasic serotonin increases the motivation to cooperate in fish (Soares, 2017). Differently from the

cleaner wrasse, however, conditional approach during predator inspection entails certain exposure to threat, because most of the clientele of *L. dimidiatus* is harmless (Côté, 2000). Acute fluoxetine increased freezing, while metergoline decreased it, suggesting that serotonin controls antipredator behaviour in guppies. Similar observations have been made in zebrafish, in which serotonin appears to have a “dual role” in mediating responses to potential vs. real threat (Herculano & Maximino, 2014).

While phasic serotonin appears to motivate cooperation in guppies, we found no evidence of a serotonergic tone either for increasing or inhibiting conditional approach. This is not consistent with other findings from the literature; for instance, in humans, tryptophan depletion seems to reduce cooperation in a Prisoner’s Dilemma game (Wood et al., 2006), and blocking 5-HT_{1A} receptors or eliminating 5-HT tonus decreases cleaning in wrasses (Paula et al., 2015). In this last case, reducing 5-HT activity also decreased cheating frequencies due to an overall reduction of the proportion of clients inspected and of the average duration of interactions (Paula et al., 2015). These effects may occur via mediation of risk perception, which would enhance the cleaner’s appraisal of the threat represented by predatory clients (Soares, 2017; Soares, Cardoso, et al., 2018; Soares, Gerlai, et al., 2018; Soares et al., 2016). These results suggest that the role of serotonin in defensive behaviour and cooperation during predator inspection are segregated, with phasic increases in both cooperation and fear, but tonic effects only on fear.

Overall, the results reported here demonstrate that serotonin acts to promote cooperation in a laboratory setting in fish. These results contribute to the description of

the neurochemical bases of pro-social behaviour, which may have translational implications. Moreover, our findings also point to a stronger relationship between fear and sociality, an area seldomly explored in the social behaviour literature (Soares, Cardoso, et al., 2018; Soares, Gerlai, et al., 2018). Whether both conditional approach and freezing are also modulated by mediators of fear and stress, such as cortisol, is still unknown.

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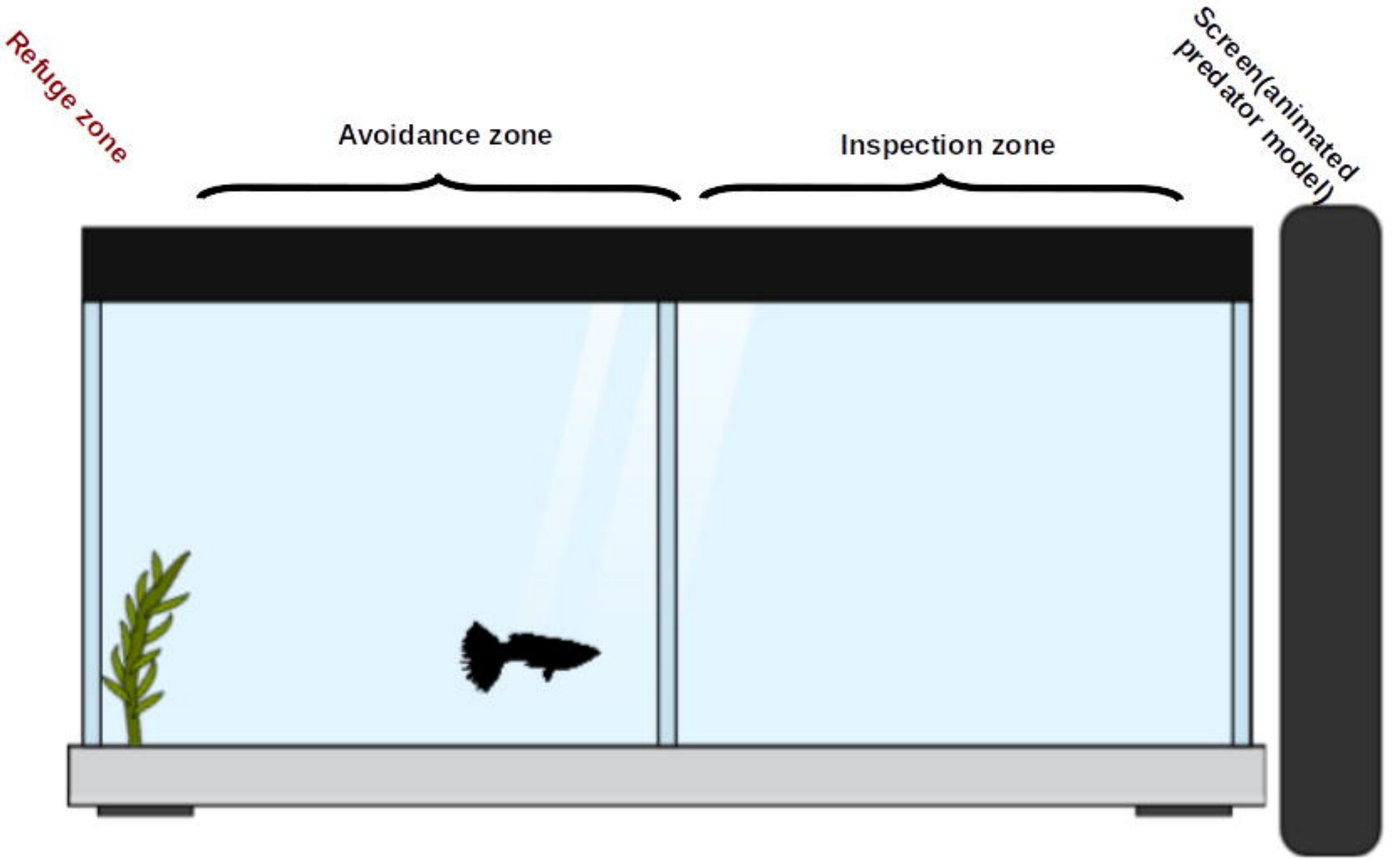
Figure captions

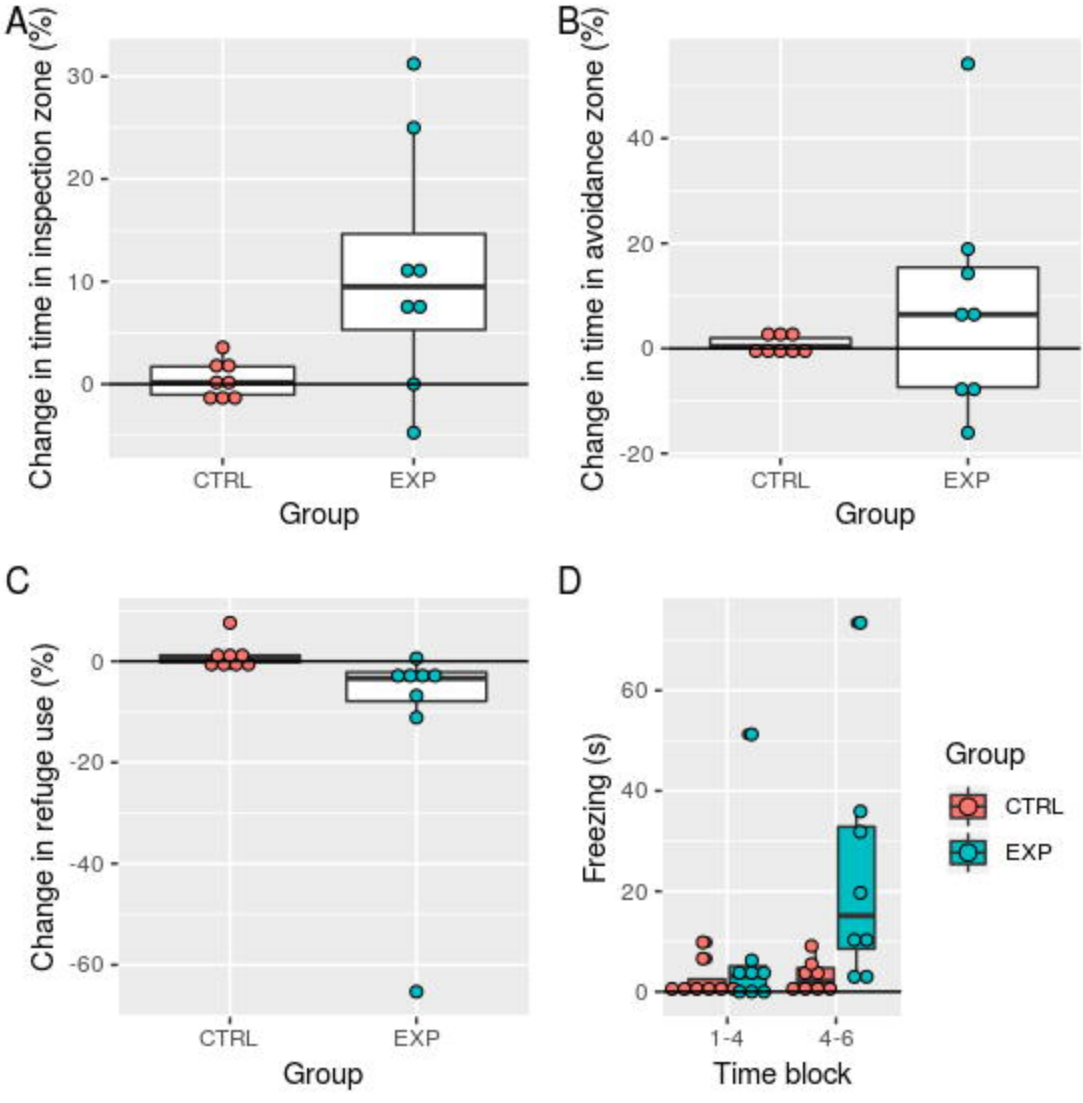
Figure 1 – Experimental setup. A glass tank (40.6 cm x 18 cm x 25 cm) was used, containing an artificial plant in one extreme ("refuge zone"), and a computer screen positioned on the opposite extreme (in which the predator animation was shown). A mirror was positioned in parallel to one of the sides of the tank. The avoidance zone is defined as the half of the tank that is farthest from the screen, and the inspection zone is defined as the other half of the tank.

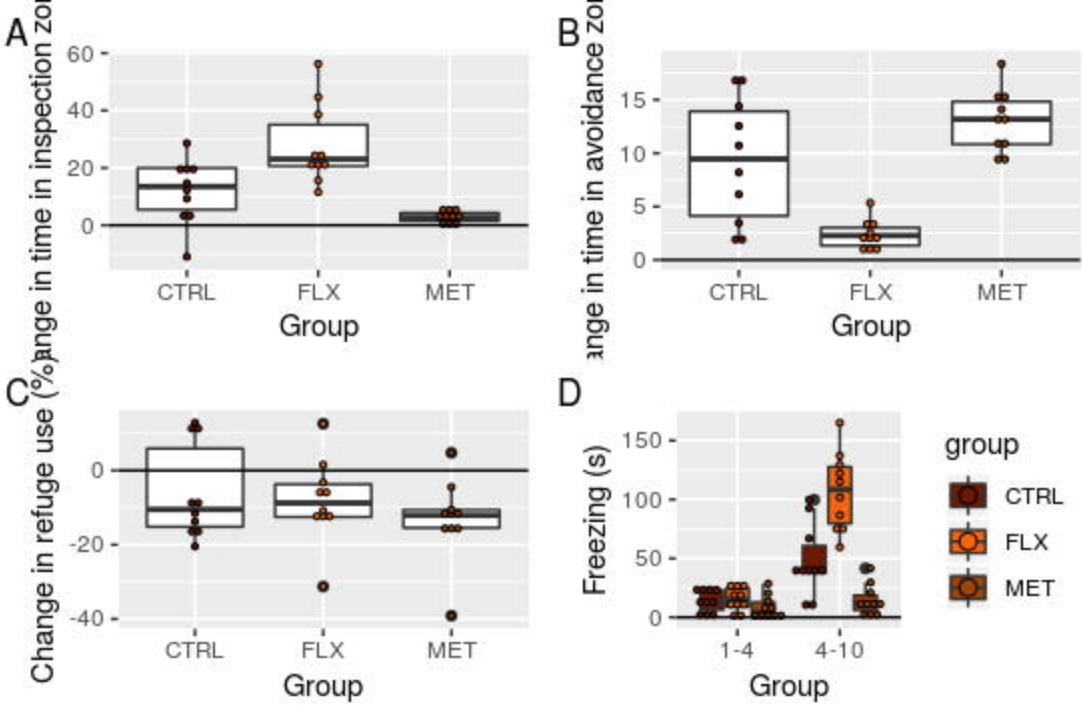
Figure 2 – Experiment 1: Conditional approach in guppies. Animals exposed to the animated image of a predator (EXP) spent more time in the inspection zone (A), but not in the avoidance (B) or refuge (C) zones of the tank, when compared to animals not exposed to the image (CTRL). The predator image also elicited freezing in EXP animals (D). Boxplots represent median ± interquartile range (IQR), with whiskers extending up to 1.5xIQR.

Figure 3 – Experiment 3: Role of serotonin in conditional approach in guppies. Animals treated with fluoxetine(FLX), but not with metergoline (MET) spent more time in the inspection zone (A) and less time in the avoidance (B) zone, but not in the refuge (C) zone of the tank, when compared to animals treated with Cortland's salt solution (VEH). Fluoxetine also increased freezing, while metergoline decreased it (D). In all groups, animals were exposed both to the parallel mirror and the animated predator image, like

the EXP group from Experiment 1. Boxplots represent median ± interquartile range (IQR), with whiskers extending up to 1.5xIQR.


Refuge zone
Avoidance zone
Inspection zone
Screen(animated predator model)


A
Change in time in inspection zone (%)
30
20
10
0
CTRL
EXP
Group
B
Change in time in avoidance zone (%)
40
20
0
-20
CTRL
EXP
Group
C
Change in refuge use (%)
0
-20
-40
-60
CTRL
EXP
Group
D
Freezing (s)
60
40
20
0
1-4
4-6
Time block
Group
CTRL
EXP


A
ange in time in inspection zo
60
40
20
0
CTRL
FLX
MET
Group
B
ange in time in avoidance zo
15
10
5
0
CTRL
FLX
MET
Group
C
Change in refuge use (%)
0
-20
-40
CTRL
FLX
MET
Group
D
Freezing (s)
150
100
50
0
1-4
4-10
Group
group
CTRL
FLX
MET