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

Ana Flávia Nogueira Pimentel[1,2], Tamires dos Santos Carvalho[2], Fernando Lima[2], Monica Lima-Maximino[3], Marta Candeias Soares[4], Caio Maximino[1,2*]

[1] Laboratório de Neurociências e Comportamento "Frederico Guilherme Graeff", Universidade Federal do Sul e Sudeste do Pará, Marabá/PA, Brazil

[2] Faculdade de Psicologia, Universidade Federal do Sul e Sudeste do Pará, Marabá/PA, Brazil

[3] Laboratório de Neurofarmacologia e Biofísica, Universidade do Estado do Pará, Marabá/PA, Brazil

[4] CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, Vairão, Portugal

* Corresponding author

Caio Maximino

Laboratório de Neurociências e Comportamento – Instituto de Estudos em Saúde e Biológicas, Universidade Federal do Sul e Sudeste do Pará, Unidade III

Av. dos Ipês, S/N, s/ CEP, Bairro Cidade Jardim, Marabá/PA, Brazil

cmaximino@unifesspa.edu.br

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 approaches the predator in the first move and subsequently approaches it only if a second individual swims even with it during inspection. This strategy is analogous to the “tit-for-tat” strategy of the Prisoner’s Dilemma, suggesting that it could be used to study cooperation. Serotonin is thought to mediate cooperative behavior in other fish species. Exposure to the animated image of a predator in a tank that contained a parallel mirror – mimicking a equally cooperating conspecific – promoted inspection and decreased refuge use, but increased 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 (2.5 mg/kg) or metergoline (1 mg/kg), and tested then in a predator inspection paradigm. Fluoxetine increased the time the animal spent inspecting the predator image, while metergoline decreased it. Fluoxetine also decreased time spent avoiding the predator and increased freezing, while metergoline decreased freezing. These results suggest that phasic increases in serotonin levels promote conditional approach, suggesting a role for this neurotransmitter in cooperation.

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1. 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 et al. 1999) and sometimes even to provide information regarding own physical condition (e.g. leaping in artiodactyls; Caro and 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; Dugatkin 1991; Dugatkin 1992) and sticklebacks *Gasterosteus aculeatus* (Milinski 1987; Huntingford et al. 1994), 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 (Dugatkin 1997; Bshary and Oliveira 2015). Conditional approach represents a cooperative strategy that is comparable to a Prisoner's Dilemma game (Luce and Raiffa 1957; Axelrod and Hamilton 1981), in which cheating a partner (by staying behind) is the most profitable option while joint inspection is the most beneficial (symmetrical) form of

cooperation , allowing the advantages of inspecting the predator to overcome those of remaining at a safe distance (Dugatkin 1997). Trivers (1971) proposed the Prisoner's Dilemma as a mechanism to interpret the evolution of reciprocal altruism, and Axelrod and 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. TIT-FOR-TAT requires a pre-specified payoff that is, in the case of conditional approach, unknown (Dugatkin 1988); however, conditional approach is analogous to TIT-FOR-TAT in that when fish inspect in pairs, due to the higher risk of taking the lead, exchanges in leading position are an example of cooperation based on reciprocity (Bshary 2001). Prisoner's Dilemma therefore models a situation in which each individual receives a worse pay-off for not cooperating than for cooperating (Trivers 1971; Axelrod and Hamilton 1981; Bshary and Oliveira 2015). 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. Conditional approach is only employed by inspecting individuals in cooperative partnerships; predator inspection *per se* can be made by solitary individuals, as has been shown for guppies (Magurran and Seghers 1990; Dugatkin and Godin 1992b) and zebrafish (Dugatkin et al. 2010). Predator inspection by singletons is still cooperative, as the rest of the shoal can benefit from the transmission of information about the predator, but involves inspection only by one individual (Dugatkin and Godin 1992a, 1992b).

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 (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; Dugatkin 1991; Dugatkin and Alfieri 1991; Dugatkin 1992; Dugatkin and Alfieri 1992). 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 and Maximino 2014; Winberg and Thörnqvist 2016; Soares, Gerlai, et al. 2018), and has been implicated in vertebrate social behaviour (Insel and 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 et al. 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 responses given previous mutually cooperative behaviour (Wood et al. 2006). In cleanerfish *Labroides dimidiatus*, acute treatment with fluoxetine or the 5-HT_{1A} receptor agonist (±)-8-Hydroxy-2-(dipropylamino)tetralin (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 N-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinylcyclohexanecarboxamide (WAY

100,635) decreased client inspection and cleaners' cheating levels (Paula et al. 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 et al. 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 (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 an equally cooperating conspecific, with approaches and retreats to the predator –, 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, 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 zone, and decrased time in the refuge zone. 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.

2. Methods

2.1. 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. The animals were bred at the vendor, and represent the third generation from populations captured in the wild in the Amazon (Belém/PA, Brazil). These domesticated animals represent a mix of strains. Animals were fed daily with commercial feed. The animals were housed collectively in 40 L tanks, at a maximum of 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.

2.2. 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, minus the refuge zone, and the inspection zone is defined as the other half of the tank (Figure 1)**.** The avoidance zone, therefore, did not include the refuge. While the mirrored image does not respond in the way a live individual would (i.e. there is not exchange of the leading position, which is the position entailing more risk), and therefore the mirrored image can only be as cooperative as the focal individual, using a

mirror to simulate cooperation has been shown to work and in fact reflect the cooperativeness of a specific individual, despite the limitations in mimicking a natural behavior (Milinski 1987; Dugatkin 1988).

Conditional approach was induced by playback of an animated predator (Acará cichlid, *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. The animals are expected to respond to acará cichlids with antipredator behavior, which has been observed in other populations (e.g., Botham et al. 2006).

2.3. 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).

2.4. Experiment 1

2.4.1. 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:

$$\frac{Time\,spent\,at\,zone\,for\,the\,last\,5min - Time\,spent\,at\,zone\,for\,the\,first\,5min}{Time\,spent\,at\,zone\,for\,the\,first\,5min} \times 100\%$$

These values were compared using independent 2-group Mann-Whitney's U tests, with *p*-values < 0.05 considered statistically significant. Effect sizes for these variables were reported as Cohen's *d*. Raw values for the acclimation period were also analyzed, to understand whether there were biases or baseline differences across groups that could be responsible for possible effects.

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.

2.5. Experiment 2

2.5.1. Drug treatments

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 et al. 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 (Kinkel et al. 2010). Briefly, animals were anesthetized in cold water (12 ºC - 17 ºC), and transferred to a “surgical bed” made of a washing sponge with a through that allowed positioning the animal with the ventral side exposed, while simultaneously perfusing the gills. 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.

2.5.2. 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. The dose was based on the average weight of the animals. 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 Kruskal-Wallis’ rank

sum test followed by Dunn's post-hoc multiple comparison tests adjusted with the Benjamini-Hochberg method; freezing 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.

3. Results

3.1. Experiment 1

No differences were found in baseline (pre-animation) values across groups on time in the refuge (W = 23, p = 0.3706; data not shown), avoidance zone (W = 29, p = 0.7984; data not shown), or inspection zone (W = 40, p = 0.4418; data not shown). The animated predator increased time in the inspection zone when the mirror was parallel to the tank (Figure 2A; W = 13, p = 0.0498, *d* = -1.25). The animated predator did not change time spent in the avoidance zone (Figure 2B; W = 24, p = 0.4418, *d* = -0.49). Refuge use was altered by the animated predator (Figure 2C; W = 59, p = 0.003, *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, ω^2 = 0.138 for the main effect of group).

3.2. Experiment 2

Acute fluoxetine increased time spent in the inspection zone (Z = -2.16, p = 0.046 vs. control), while metergoline decreased time in the inspection zone (Z = 2.01, p = 0.045 vs. control)(Figure 3A; $\chi^2_{[df = 2]}$ = 17.36, p = 0.00017, ω^2 = 0.476). Only

fluoxetine produced an effect on time spent in the avoidance zone (Figure 3B; $\chi^2_{[df = 2]} = 16.059$, $p = 0.00033$, $\omega^2 = -0.555$), with fluoxetine decreasing time spent in the avoidance zone ($Z = 2.616$, $p = 0.013$ vs. control), and metergoline having no effect ($Z = -1.32$, $p = 0.186$ vs. control). Neither fluoxetine nor metergoline altered refuge use (Figure 3C; $\chi^2_{[df = 2]} = 1.319$, $p = 0.517$, $\omega^2 = -0.00331$). 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).

4. 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, and limited 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, as well as time spent in the avoidance and refuge zones. While freezing and refuge use were indeed increased, animals did not appear to avoid the predator. We suggest that freezing, in this situation, is an optimal strategy to avoid being detected by the predator while at the same time benefiting from gathering information, maintaining a high level of alertness. The relationship between fear, stress, and social behaviour has seldomly been

addressed in the literature (Soares, Cardoso, et al. 2018; Soares, Gerlai, et al. 2018). There is some evidence from cleaning gobies *Elacatinus* spp. that cooperating in cleaning mutualisms with piscivorous clients (potential predators) is stressful, as cortisol levels are increased during these interactions (Soares et al. 2012). Interestingly, field experiments in *L. dimidiatus* showed that treament with cortisol increases “cheating”, in which cleaners provide small clients with more tactile stimulation with their pectoral and pelvic fins – a behaviour that attracts larger clients that are then bitten to obtain mucus – , while blocking glucocorticoid receptors led cleaners to increase tactile stimulation to larger clients (Soares et al. 2014).

An important concern of our experiments is that, differently from Dugatkin (1988), the animals used in Experiments 1 and 2 are not wild-caught, but laboratory-reared, and the stimulus predator was the blue acará cichlid. As a result, animals could have been displaying behavior that is not specifically anti-predator in nature, but instead represent a “curiosity” approach to any novel fish stimulus. Indeed, in wild populations from Trinidad, Pike cichlids (*Crenicichla lepidota*) induce a “surfacing” behavior that is not observed when animals are exposed to acará cichlids (Botham et al., 2006); however, even in wild populations, acará and Pike cichlids do not differ in their ability to induce an inspection response or in their ability to induce fear-like behavior (e.g., inhibit foraging and increase shoaling) (Botham et al., 2006). While wild-caught female guppies from high-predation areas appear to respond less to acará cichlids than to Pike cichlids, this difference disappears at the first and second generations derived from these wild populations (Kelley and Magurran, 2003). In the light of these findings, and considering the effect that our acará cichlid animation had on freezing, our results suggest that this domesticated population is displaying normal antipredator behavior, and not simply a novelty response. However, a neophobic response cannot be fully discarded as driving

part of the behavior reported here.

An alternative explanation to the results from Experiment 1 is that predator inspection does not represent conditional approach, but instead males undertake inspection as a demonstration to females of their superiority in relation to non-inspectors. Indeed, it has been demonstrated that female guppies prefer bolder males, which show higher levels of inspection (Godin & Dugatkin, 1996; Croft et al., 2006;), and bolder individuals tend to produce more social interactions (Croft et al., 2009); assortative interactions and sexual selection are expected to play a role in the establishment of this strategy (Edenbrow et al., 2017). However, this is not necessarily contradictory with the conditional approach strategy since, differently from “pure” TIT-FOR-TAT, conditional approach does not require some form of kin or group selection to be at work. Conditional approach can be better understood in a “social competence” approach (Bshary and Oliveira, 2015), in which situation-specific cues (such as presence or absence of females, or varying risk levels), together with various other factors, allow individuals to assess the situation in order to make behavioral decisions. While risk taking certainly takes part on this (Dugatkin, 2013), it is not the only variable responsible for the development of predator inspection in our paradigm.

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 fear, while acute metergoline decreased both. 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 probability of cleaning 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 exposure to threat, while most of the clientele of *L. dimidiatus* is harmless. 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 and Maximino 2014).

While phasic serotonin appears to motivate cooperation in guppies, we found evidence of a serotonergic tone promoting conditional approach. This is consistent with other findings from the literature: tryptophan depletion reduces cooperation in the Prisoner’s Dilemma in human participants (Wood et al. 2006), and 5-HT_{1A} receptor antagonism 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 et al. 2016; Soares 2017; Soares, Cardoso, et al. 2018; Soares, Gerlai, et al. 2018).

5. Conclusion

Overall, the results reported present a scenario in which 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, and could have translational applications. Moreover, they also shed light on the 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 this is also modulated by mediators of fear and stress, such as cortisol, is still unknown.

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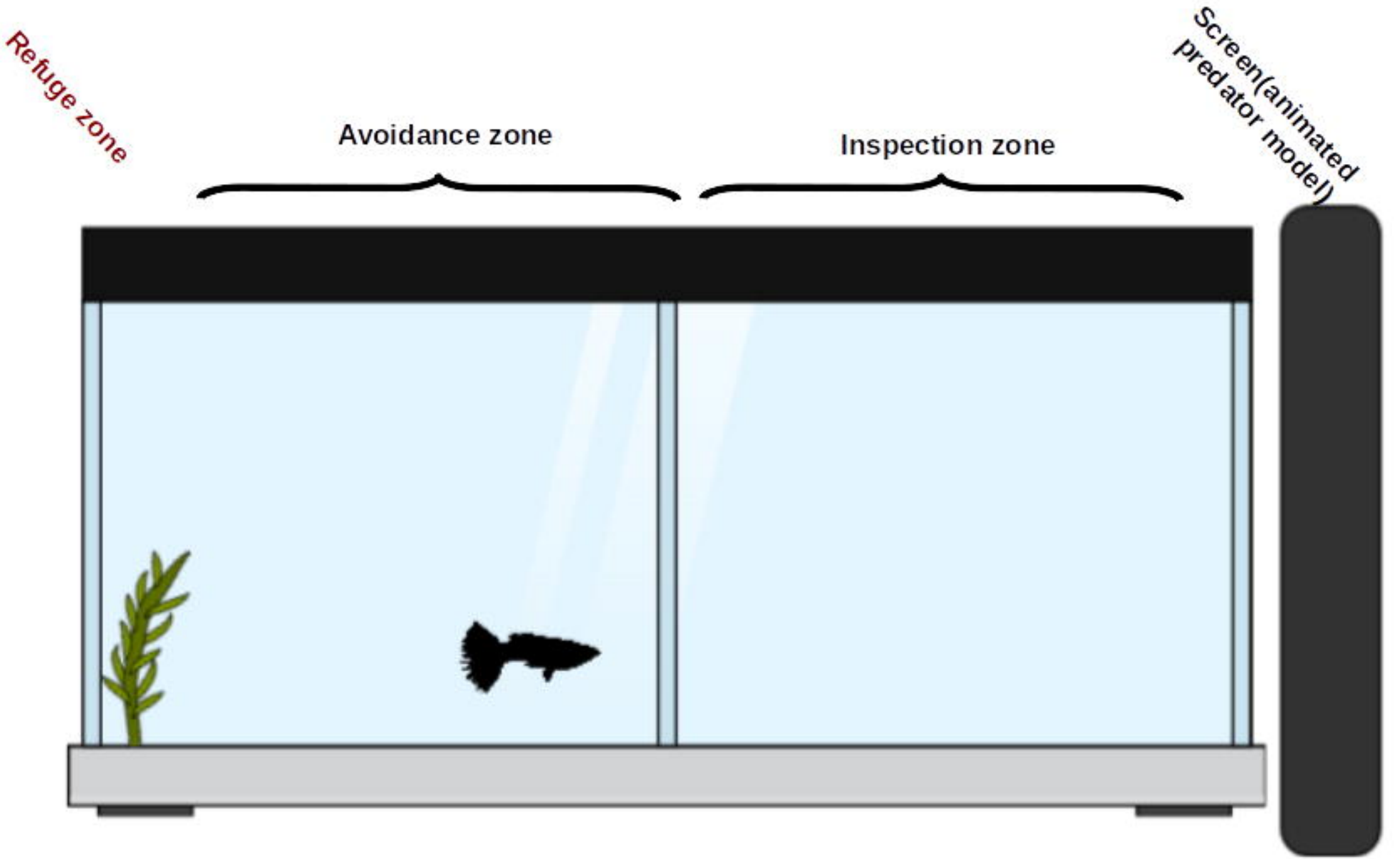
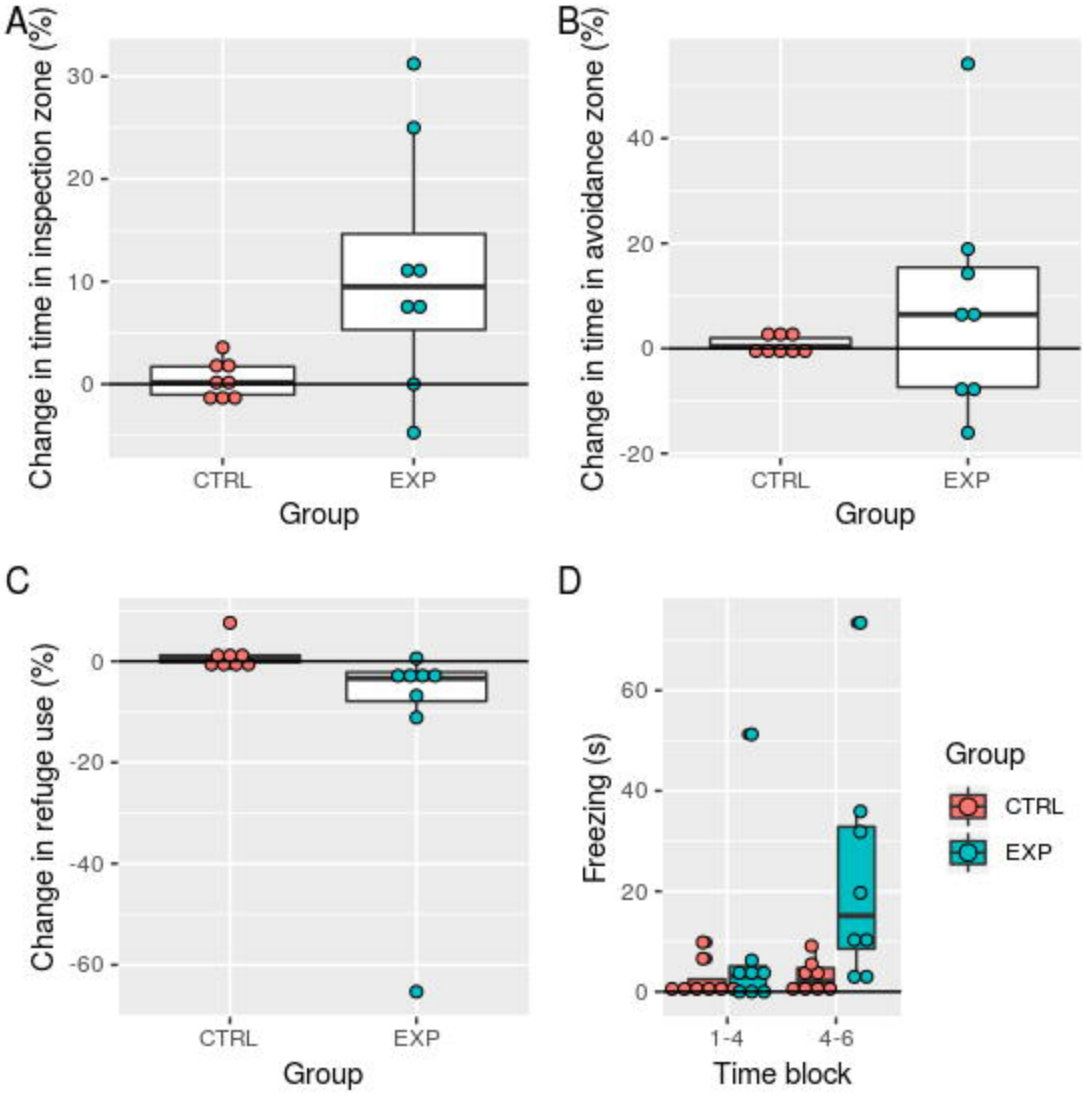
Figure captions

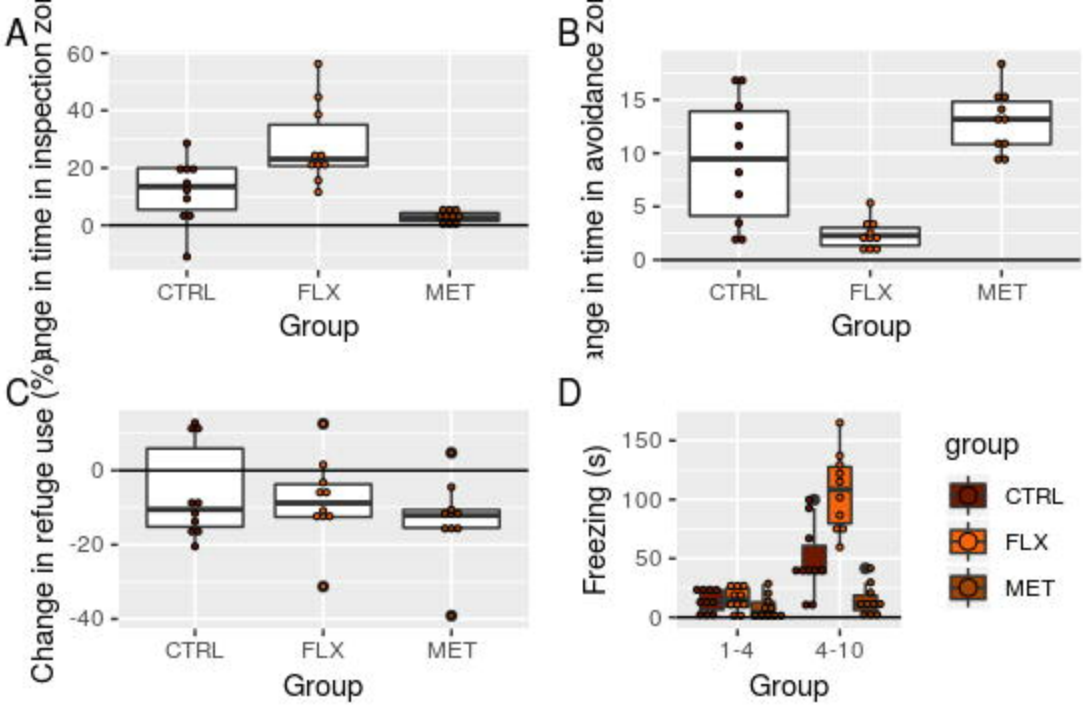
Figure 1 – Experimental setup. A glass tank (40.6 cm x 18 cm x 25 cm) was used, containing an artificial plant in one extreme ("refuge zone"), and a computer screen positioned on the opposite extreme (in which the predator animation was shown). A mirror was positioned in parallel to one of the sides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