Cannabidiol improves fish welfare

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Abstract

Cannabidiol (CBD) is a substance derived from Cannabis sativa, widely studied in medicine for controlling neural diseases in humans. Besides the positive effects on humans, it also presents anxiolytic proprieties and decreases aggressiveness and stress in mammals. Therefore, CBD has the potential to increase welfare in reared animals, as it seems to reduce negative states commonly experienced in artificial environments. Here, we tested the effect of different CBD doses (0,1,10, and 20 mg/kg) on aggressiveness, stress, and reproductive development of the Nile tilapia (*Oreochromis niloticus*) a worldwide fish reared for farming and research purposes. CBD mixed with fish food was offered to isolated fish for 5 weeks. The 10 mg/kg dose decreased fish's aggressiveness over time, whereas 20 mg/kg attenuated non-social stress. Both doses decreased the baseline cortisol level of fish and increased the gonadosomatic index. However, CBD 1 and 10 mg/kg doses decreased the spermatozoa number. All CBD doses did not affect feeding ingestion and growth variables, showing that it is not harmful to meat production amount. Despite the effect on spermatozoa, CBD supplementation exhibits high potential to benefit animals' lives on an integrative-based welfare approach. Therefore, we showed for the first time that CBD could be used as a tool to increase non-mammal welfare, presenting a great potential to be explored in other husbandry and captivity species.

Introduction

The science of animal welfare has been growing in this century, as several studies showed the importance of meeting the animal needs to ensure them a better quality of life[1–3]. Thus, researchers have been focused on investigating means of mitigating the effects of artificial environments by reducing stress and stimulating positive states in animals.

Despite many studies regarding animal welfare, they usually fall within one of the following three approaches. The "natural-living approach" considers that animals are at a good welfare level when they are able to express, in captivity, those behaviors that they would perform in natural environments[2,4]. The "affective state approach" assumes that animals need to be free of suffering, intense and prolonged pain, fear, hunger and other negative affective states[4,5]. Finally, the "functional approach" takes into account that being at a good welfare level means that animals must have a good biological system's functionality and good health when they are coping with their environments[4,6]. However, Fraser et al.[4] suggested that these three concepts are intrinsically connected and that the best understanding of animal welfare will address the concerns of these three approaches. The precepts of this integrative approach are gradually being included in animal farming, such as cattle and poultry[2,7]. However, this integrative approach is still incipient in fish rearing environments[3].

In aquaculture, fish are usually exposed to several stressors that affect their welfare, such as changes in water quality[8], handlings (e.g., grading, capturing, and transporting[9]), and high stock densities[6,10]. Overall, these stressors will trigger physiological responses such as activation of the autonomic nervous system following rapid cardiac and respiratory adjustment [ventilation rate (VR) increase, for example], and

epinephrin releasing, as well as activation of the Hypothalamus-Pituitary-Interrenal axis, culminating in increased cortisol levels[6,11] (functional approach). When prolonged, these physiological responses can lead animals to non-adaptive behavioral and morphological alterations, such as decreasing reproductive performance[9,12], reducing food intake, weight loss, and impairment of the immune system[6,11]. In particular, the high stock density in aquaculture can increase the number of aggressive interactions in hierarchical and territorial species[13,14].

Although aggressive interactions are a natural component of many fish species' behavior, the rearing environment can increase this type of behavior to non-natural levels (natural-living approach)[14]. The higher the aggressive interactions, the higher the social stress from social rank, the energy expenditure of fights, and the probability of body injuries[14], being this last one a source of pain (affective state approach). Thus, the aquaculture rearing conditions can exacerbate fish aggressive behavior and shrinks its adaptive value[14]. Altogether, these behavioral and physiological alterations triggered by the aquaculture environments compromise fish welfare from the point of view of an integrative approach. Therefore, finding strategies that improve fish welfare and mitigate as many as possible adverse effects of the rearing environment is of great importance.

A way to counteract the negative effects of the rearing environment in fish is by using chemical substances known to reduce stress and stimulate indicators of good welfare, usually those acting on the central nervous system. For example, the amino acid tryptophane mixed in the food reduces aggressive behavior and stress in Nile tilapia (*Oreochromis niloticus*) (Linnaeus, 1857)[15] and rainbow trout (*Oncorhynchus mykiss*[16,17]), probably by increasing serotonin levels or activating serotoninergic pathways in the brain[16,17]. Recently, a promising substance that has a great potential to increase the welfare of farm animals is one of the major cannabinoids from the Cannabis sativa plant, the cannabidiol (CBD)[18]. The CBD presents many pharmacological properties and great medicinal potential, assisting the treatment of many human diseases and psychiatric disorders[19]. In non-human mammals, the CBD shows anxiolytic-like[20,21] and antidepressant-like effects[22,23], decreases the aggressiveness[24,25] and the stress[26,27], has anti-inflammatory effects[28], benefits the food intake and weight gain[29], and regulates the fertility[30].

The mechanisms responsible for most CBD effects are still not completely elucidated. CBD acts in multiple targets and receptors, such as the serotoninergic system, by activating 5-HT1A receptors[24,31], and the endocannabinoid system. Essentially, this is a neuromodulator system, which will allow or ceases the neurotransmissions throughout the organism[32]. CBD acts indirectly on this system, blocking the fatty acid amide hydrolase enzyme (FAAH)[30,32], responsible for degrading anandamide, one of the primary vertebrates endocannabinoids ligands[33]. Thus, CBD increases the anandamide supply in organisms and consequently increases the activation of CB1 and CB2 endocannabinoid receptors[24].

The CBD decreases aggressiveness in mammals through a mechanism associated with activating both receptors, 5-HT1A and CB1[24]. The activation of 5-HT1A receptors by the drug is also related to the

decrease in stress and anxiety[27,34]. The endocannabinoid receptors also have a regulatory effect on the hypothalamic-pituitary-adrenal axis (HPA), responsible for mediating stress responses in mammals[35], besides having a key role in the food intake and body weight gain[36]. Moreover, the endocannabinoid system is involved in the regulation of male[37,38] and female fertility[39,40].

The effects of CBD demonstrated in mammals are expected to be similar in other vertebrate groups, such as fish, since the 5-HT1A serotoninergic receptors and the endocannabinoid system are highly conservated between the taxa[41]. For example, fish CB1 receptors have about 70% similarity with CB1 receptors of rodents and humans[42]. Thus, it is expected that the CBD effects in mammals involving the activation of these two receptors are similar in fish. Indeed, some studies have already shown that in zebrafish (Danio rerio), CBD presents an anxiolytic effect[43]; decreases the natatory rhythm, stimulates differentiation and regulation of immunity genes[44]; and improves some reproductive parameters in females, although showing reproductive toxicity for males[38]. These studies show the potential of CBD to improve fish welfare in captivity environments, mainly in aggressive species. Here, we tested the effect of CBD on aggressiveness, stress and reproductive development in Nile tilapia, a cichlid fish species highly important for aquaculture. Since CBD's effects vary according to the dose[45], we tested doses of 0 (control),1 (CBD 1),10 (CBD 10), and 20 mg/kg (CBD 20), which were mixed with food and offered to fishes for 5 weeks (Fig. 1). We predicted that high doses of CBD would increase fish welfare by decreasing animals' aggressiveness and stress responses (specifically, VR and cortisol levels) and improving reproductive aspects, such as testes size and spermatozoa number.

Results

A high dose of CBD reduced aggressive behavior

The same test (mirror test - see Methods for more details) was used during the first four experimental weeks to assess fish's aggressive behavior and stress response to a social stimulus (fish's reflected image in the mirror) (Fig. 1). These responses were collected through five sampling time points: basal (the first day of experiment, before fish start to receive the treated diets) and once a week after the beginning of CBD treatment (week 1, week 2, week 3 and week 4; Fig. 1). To assess fish's aggressive behavior, the frequency of direct attacks and the latency for the first aggressive behavior against the mirror were accounted for (see Methods for more details about the "Mirror Test").

Fish from the CBD 10 treatment significantly decreased attacks over the sampling time points (Fig. 2a). During the third and fourth weeks of CBD administration, fish from CBD 10 treatment attack less the mirror than in their basal measurement. This decrease in the number of attacks was not observed in the other treatments [Linear mixed model (LMM), treatment: $_{F3, 55.94} = 0.362$, $p = 0.78$; sampling time points: $F_{4, 222.1} = 2.516$, $p = 0.042$; treatment * sampling time points: $F_{12, 222.11} = 2.424$, $p = 0.006$; Tukey HSD test: CBD10 basal vs. week 3: $p < 0.001$; CBD 10 basal vs. week 4: $p < 0.001$; Fig. 2a].

Regarding the latency for the first aggressive behavior, fish from the control treatment decreased the latency for their first attack against the mirror over the time (Fig. 2b). In the third week, fish from the control group performed their first attack significantly faster than their basal measurement. This pattern was not observed in any other treatment exposed to CBD [LMM, treatment: $F_{3, 56.266} = 1.253$, $p = 0.299$; sampling time points: $F_{4, 222.638} = 3.03$, $p = 0.018$; treatment * sampling time points: $F_{12, 222.644} = 1.986$, $p = 0.027$; Tukey HSD test: control basal vs. week 3: $p = 0.042$; Fig. 2b].

CBD did not attenuate stress in response to a social stimulus

We tested whether different CBD doses could attenuate fish' stress responses induced by their reflected image on a mirror (social stimulus)[46]. For this purpose, we measured animals' ventilation rate (VR) before (pre-social stimulus) and after (post-social stimulus) the mirror test (Fig. 1). In addition, we also measured the individual variation of VR in response to the mirror test (ΔVR = VR post-social stimulus– VR pre-social stimulus) (see Methods for more details about the VR measurement).

In general, we only observed significant short-term alterations in VR in the first week (Fig. 3). All these differences did not persist during the other weeks. Regarding the VR pre-social stimulus, in the first week it was significantly higher in CBD 10 treatment compared to control and CBD 1 groups [LMM, treatment: $F_{3, 60} = 0.589$, $p = 0.624$; sampling time points: $F_{4, 240} = 1.785$, $p = 0.132$; treatment * sampling time points: $F_{12, 240} = 3.742$, $p < 0.001$; Tukey HSD test: week 1 CBD 10 vs. Control: $p < 0.01$; week 1 CBD 10 vs. CBD 1: $p = 0.045$; Fig. 3a, left graph]. Moreover, in the week 1, VR of CBD 10 treatment was significantly higher compared to their basal and week 4 measurements [Tukey HSD test: CBD 10 week1 vs. Basal: $p = 0.045$; CBD 10 week1 vs. week 4: $p < 0.01$; Fig. 3a, right graph]. Further, in the week 1, VR pre-social stimulus of control treatment was significantly lower compared to their basal and week 3 measurements (Tukey HSD test: Control week 1 vs. Basal: $p = 0.045$; Control week 1 vs. week 3: $p = 0.037$; Fig. 3a, right graph).

Similarly, the VR post-social stimulus was significantly affected only in week 1 (Fig. 3b). In week 1, fish from CBD 10 treatment presented a higher VR compared to control and CBD1 treatments within the same sample time point (LMM, treatment: $F_{3, 60} = 1.346$, $p = 0.268$; sampling time points: $F_{4, 240} = 4.529$, $p = 0.001$; treatment * sampling time points: $F_{12, 240} = 4.503$, $p < 0.001$; Tukey HSD test: week 1 CBD 10 vs. Control: $p = 0.028$; week 1 CBD 10 vs. CBD 1: $p < 0.01$; Fig. 3b, left graph). Moreover, in week 1 CBD 10 fish increased their VR compared to their basal, week 3 and 4 measurements [Tukey HSD test: CBD 10 week1 vs. Basal: $p = 0.019$; CBD 10 week1 vs. week 3: $p < 0.01$; CBD 10 week1 vs. week 4: $p < 0.01$ Fig. 3b, right graph]. On the other hand, in the first week, control and CBD 1 treatments decreased their VR compared to their respective basal measurements. The VR post-social stimulus of control group in week 1 was also lower than their measurement in week 3 [Tukey HSD test: Control week 1 vs. Basal: $p < 0.01$; Control week1 vs. week 3: $p < 0.01$; CBD 1 week1 vs. Basal: $p = 0.015$; Fig. 3b, right graph].

In relation to the ΔVR we did not observe a significant effect of treatments, sampling time points, neither interaction between treatments and sampling time points (LMM, treatment: $F_{3, 60} = 1.929$, $p = 0.134$;

sampling time points: $F_{4, 240} = 0.867$, $p = 0.484$; treatment * sampling time points: $F_{12, 240} = 0.62$, $p = 0.824$).

High CBD dose reduced fish's VR increase in response to a non-social stimulus

Even did not attenuate stress from a social stimulus, in the fifth week of CBD administration, we tested if the drug could attenuate fish' stress responses (namely, VR and cortisol levels) to a confinement stressor (non-social stress) (Fig. 1). We measured animals' VR pre-confinement, VR post-confinement, and fish' VR variation (ΔVR) in response to confinement stress. In addition, we measured fish' baseline cortisol levels (without applying stress) and the stress-induced cortisol levels (after applying confinement stress in fish) (see Methods for more details about VR and cortisol).

We did not observe a significant effect of CBD on VR pre-confinement (Kruskal-Wallis, $H_3 = 4.258$, $p = 0.235$) and post-confinement (one-way ANOVA, $F_{3, 45} = 0.336$, $p = 0.799$). However, we observed a significant effect of CBD on the ΔVR. Fish from CBD 20 treatment increased less the VR after confinement stress, presenting a lower ΔVR compared to control fish (one-way ANOVA, $F_{3, 45} = 3.08$, $p = 0.037$; Tukey HSD test: CBD 20 vs. Control: $p = 0.045$; Fig. 4).

High CBD doses decreased the baseline but not the stress-induced cortisol levels

The baseline cortisol levels of fish from CBD 10 and CBD 20 groups were lower than control group (one-way ANOVA, $F_{3, 24} = 7.621$, $p < 0.001$; Fig. 5; Tukey HSD test: CBD 10 vs. Control: $p = 0.023$; CBD 20 vs. Control: $p < 0.01$; Fig. 4). However, in relation to the stress-induced cortisol levels, we did not observe a significant effect of CBD (one-way ANOVA, $F_{3, 27} = 1.127$, $p = 0.356$).

CBD did not affect feeding ingestion and growth variables

During the acclimatization week and the first four experimental weeks, fish feed ingestion and the following growth variables were measured: final standard length, final weight, average weight gain (AWG), feed conversion (FC), specific growth rate (SGR) and condition factor (K). In addition, feeding ingestion was also measured in the fifth week and analyzed apart from other weeks due to fish manipulation for blood sample collection (see Methods for more details).

The feed ingestion during the acclimatization week did not differ between the treatments ($H_3 = 5.2363$, $p = 0.155$). Moreover, CBD did not decrease fish feed ingestion during the first four experimental weeks. Independent of the CBD dose received, fish decreased their feed ingestion in the last experimental weeks (LMM, treatment $F_{3, 60} = 0.641$, $p = 0.591$; sampling time points: $F_{3, 180} = 5.304$, $p = 0.002$; treatment * sampling time points: $F_{9, 180} = 0.892$, $p = 0.533$; Tukey HSD test: week 1 vs. week 3: $p = 0.034$; week 1 vs.

week 4: p = 0.003; week 2 vs. week 4: p = 0.029). Fish feeding ingestion was also unaffected by CBD in the fifth experimental week (Kruskal-Wallis test, H_3 = 3.084, p = 0.379).

In addition, none growth variable analyzed was affected by CBD [Final standard length: one-way ANOVA, $F_{3, 55}$ = 0.233, p = 0.873; final weight: Kruskal-Wallis Test, H_3 = 1.09, p = 0.779; AWG: Kruskal-Wallis Test, H_3 = 0.37, p = 0.946; FC: Kruskal-Wallis Test, H_3 = 0.128, p = 0.988; SGR: one-way ANOVA, F_3, 55 = 0.096, p = 0.962) and condition factor (K); Kruskal-Wallis Test, H_3 = 1.757, p = 0.624; Table 1].

Table 1
Growth response variables of Niles tilapias fed during 28 days with diets containing different cannabidiol (CBD) doses. The absence of asterisks indicates there is no significant difference between the treatments under one-way ANOVA or Kruskal-Wallis tests. Values are mean ± SD (N = 15). No significant differences between the treatments under one-way ANOVA or Kruskal-Wallis tests.

Treatments	Final standard length (cm)	Final weight (g)	AWG[a] (g)	FC[b] (g/g)	SGR[c] (%)	K[d] (%)
Control	11.04 ± 0.697	47 ± 9.024	17 ± 7.512	1.742 ± 0.895	1.242 ± 0.492	3.483 ± 0.484
CBD[e] 1	11.043 ± 0.746	47.667 ± 7.761	18 ± 7.746	1.737 ± 1.158	1.337 ± 0.534	3.476 ± 0.481
CBD 10	10.932 ± 0.512	45.286 ± 6.65	16.357 ± 6.935	1.707 ± 0.395	1.37 ± 0.369	3.503 ± 0.477
CBD 20	10.909 ± 0.794	45.333 ± 10.431	19.545 ± 8.409	1.681 ± 0.983	1.286 ± 0.489	3.454 ± 0.507

[a]AWG – Average weight gain. [b]FC – Feed conversion. [c]SGR – Specific Growth Rate. [d]K – Condition Factor. [e]CBD – Cannabidiol.

CBD increased testes size but decreased the number of spermatozoa

At the end of the fifth experimental week, fish were euthanized, and the testes were collected for morpho-histological analyzes (Fig. 1). Right after being collected, the gonads were weighed, and the gonadosomatic index (GI) was calculated for each fish (gonad weight proportional to the fish weight; see Methods for more details). Afterward, the testicular implants were treated, and histological slides of testes were made to account fish's relative number of spermatozoa (see Methods for more details).

Fish from CBD 10 and CBD 20 treatments presented a higher GI than those from control and CBD 1 treatments (one-way ANOVA, $F_{3, 34}$ = 11.121, p < 0.001; Tukey HSD test: CBD 10 vs. control: p = 0.004; CBD 10 vs. CBD 1: p = 0.031; CBD 20 vs. control: p < 0.001; CBD 20 vs. CBD 1: p < 0.001; Fig. 6a). Although CBD have increased fish testes size, the drug significantly decreased the number of spermatozoa (Fig. 6b-e). CBD 1 and 10 fish presented a lower number of spermatozoa by field than control fish. Moreover, fish from CBD 1 treatment presented significant less spermatozoa than any other CBD group (one-way

ANOVA. $F_{3, 13} = 18.588$, $p < 0.001$; Tukey HSD test: Control vs. CBD 1: $p < 0.001$; control vs. CBD 10: $p = 0.042$; CBD 1 vs. CBD 10: $p = 0.027$; CBD 1 vs. CBD 20: $p < 0.001$; Fig. 6b).

Discussion

This study investigated CBD effects on behavioral and morpho-physiological variables related to Nile tilapia welfare. We found that the CBD 10 mg/kg dose efficiently reduced fish aggressiveness over 28 days. Regarding stress, CBD was not efficient in mitigating the stress responses of fish induced by a social stimulus. However, the CBD 20 mg/kg dose efficiently attenuated the VR increase induced by confinement stress (non-social stressor). Moreover, in the fifth week of the experiment, fish from CBD 10 and CBD 20 treatments presented lower baseline cortisol levels. None CBD dose affected feed ingestion or any growth variable of fish. In addition, the 10 and 20 mg/kg doses increased fish GI. Although fish testes increased, doses of 1 and 10 mg/kg significantly reduced fish spermatozoa.

The CBD efficacy in decreasing animals' aggressiveness was proved only in mammals, specifically in rats and dogs[24,25], but not so far in other taxa. This study was the first to show that oral CBD administration is efficient in reducing fish aggressiveness. Proving that CBD decreases aggressiveness in multiple species is essential to translational medicine. It can help researchers identify evolutionary conservated mechanisms and shared effects of the drug among several species and focus on the overlapping feature between them[47]. Ultimately, this finding can help researchers develop CBD treatments for disorders related to aggressiveness increase in humans.

The intermediate CBD dose of 10 mg/kg was efficient in reducing fish aggressive behavior, while lower (1 mg/kg) and higher doses (20 mg/kg) were ineffective. In rats, intraperitoneal doses between 5 and 60 mg/kg of CBD decreased aggressiveness, being the doses of 15 and 30 mg/kg the most efficient[24]. The mechanism through which CBD decreases rats' aggressive behavior is associated with activating 5-HT1A serotoninergic receptors and CB1 endocannabinoid receptors[24]. Although we did not investigate the mechanism involved in CBD anti-aggressive effects in fish, it was probably similar to the mechanism described in mammals since both receptors, 5HT1-A[48,49], and CB1, are highly conservated between the taxa[41,42] and play similar roles in aggressiveness decrease[50]. The activation of the endocannabinoid system by CBD and other cannabinoids often triggers biphasic effects in behavioral responses, such as feeding, locomotor activity, and anxiety-like behavior[45]. The following scenarios characterize biphasic effects: low doses of a drug trigger an effect, while higher doses do not, or even trigger the opposite effect, and vice-versa[51]. The same classic CBD biphasic effect was probably found in our aggressive behavior results since the intermediate dose of 10 mg/kg decreased fish aggressiveness while the higher dose of 20 mg/kg did not.

In order to be in good welfare conditions, animals must express natural behaviors in captive environments[2,4]. As the Nile tilapia is an aggressive cichlid[52,53], fish must present aggressive behaviors in the stock tanks. However, environmental conditions in aquaculture tanks, mainly the high stock densities

that fish are subject to, intensify aggressive encounters to unnatural levels[14]. This raised aggressiveness level increases social stress and fish injuries, shrinking the adaptive value of aggressive behavior and reducing fish welfare[14]. Therefore, CBD 10 mixed in the ration can be a promising alternative to manage these deleterious consequences of captive environments since it can reduce fish aggressiveness and maintain it close to its natural levels.

Regarding stress, fish treated with CBD 10 increased VR pre- and post-social stimulus in the first week, which returned to baseline levels afterward (Fig. 3a and 3b). The VR is a reliable indicator of stress[54] and anxiety-like effects on fish[55,56], and although we did not perform tests to analyze anxiety, the rise of VR despite the presence of a stressor may indicate anxiogenic-like effects of CBD. In general, acute CBD administrations present anxiolytic effects[43,57]. However, some chronic short-term CBD administration (11 days) results in anxiogenic-like effects in rats[58]. Likewise, other cannabinoids, such as the HU-210, show anxiogenic effects a few days after administration, but this effect seems to be attenuated over time[35,59], as observed in our results. Thus, further studies are necessary better to understand the possible anxiogenic-like effects of initial exposure to CBD.

CDB did not attenuate VR response to social stimulus, and there may be multiple reasons for this to happen. First, the type of stressor, that is, if it is social or non-social, may influence the CBD stress-attenuation effect. In humans, a 10 mg/kg CBD dose effectively decreased the heart rate and cortisol levels in response to a non-social stimulus (drug induction[60]), while the same dose was ineffective in attenuating the cortisol increase in response to a social stressor ("trier social stress test") in patients with a high risk of psychosis[61]. Likewise, in our study, CDB was ineffective in reducing fish stress in response to a social stimulus but effective in reducing fish non-social stress (CBD 20 – Fig. 4), showing that the type of stressor may play an essential role in CBD effects. Second, it is important to highlight that the time of exposure to CBD also can be a factor that interferes with its effects[62]. The stress in response to social stimulus was measured during the four initial experimental weeks, while the non-social stress was measured in the fifth week. Thus, we cannot discard the time of exposure to CBD may also have played a key role in our results. Nevertheless, further studies are necessary to unravel the proper dosages and mechanisms behind CBD efficiency to mitigate different types of stress, primarily if the drug can mitigate social stress.

Besides the VR, we also evaluated the cortisol levels, the main stress hormone in fish[63], in the fifth week of CBD exposure, at baseline levels, and after facing confinement (stress-induced levels in response to non-social stress). The exposure to CBD did not alter the cortisol stress-induced level but substantially decreased the baseline cortisol levels in the fifth week. On the other hand, Mortuza et al.[64] found that CBD did not significantly decrease other secondary stress biomarkers, such as glucose, haematocrit, and plasma protein in both stressed and non-stressed Nile tilapias. The differences between our studies regarding the non-stressed cortisol levels are probably due to differences in the exposition time and doses. Mortuza et al.[64] exposed Nile tilapias to small doses of CBD, in which the highest dose tested was similar to our smallest dose, 1 mg/kg (See Methods - Table 2), and for a short period of 3 days.

Table 2

General information about the cannabidiol (CBD) amount applied in each treatment diet (2 kg of ration per treatment). The CBD doses of each treatment (0, 1, 10 e 20 mg/kg) were calculated based on initial fish weight. In "Initial fish weight", values are mean ± SD.

Treatments (mg of CBD[1]/ kg of fish)	Initial fish weight (kg)	CBD amount applied to diet (mg)	CBD percentage on the ration (%)
Control	0.03 ± 0.003	0	0
CBD1 (1 mg/kg)	0.02967 ± 0.003	16.5	0.0033
CBD10 (10 mg/kg)	0.02867 ± 0.002	166.5	0.0333
CBD 20 (20 mg/kg)	0.02833 ± 0.003	333	0.0666

[1]CBD – Cannabidiol.

The decrease of baseline cortisol levels is promisor to the welfare improvement of captive fish. Lower baseline cortisol levels can be beneficial to organism functioning since higher cortisol levels for long periods lead to impairment of reproduction system[65], depress immunity[66], impair the growth and organs development[67,68], among other negative effects in the organism[68]. However, it is also important to highlight the adaptative effects of cortisol, a hormone responsible for allowing animals to cope with environmental challenges, improving survival and fitness[11]. Thus, even though baseline cortisol was reduced, fish increased their cortisol levels like those not submitted to CBD after an acute stressor (stress-induced cortisol level, $p = 0.412$), showing the natural physiological and behavioral responses to coping with a stressor. These physiological responses are important from an integrative welfare perspective since CBD may improve fishes' organism functioning in rearing conditions, but at the same time, it is not deleterious for fish when facing environmental challenges.

Besides playing a role in the aggressiveness and stress axis[69], the endocannabinoid system is related to regulating food intake and body weight gain[69], and cannabinoids such as CBD may affect those factors[29,36]. Studies diverge on whether CBD can increase[29], not affect[70], or even decrease body weight gain[36]. Probably, these differences are because the drug's effects depend on many factors such as dose, animals' stress level, taxon, sex, and others. In our results, juvenile males of Nile tilapia supplemented with different CBD doses did not alter food intake or any of the measured growth response variables. It is important to highlight that diet supplemented with CBD was not unpalatable for fish, and the method of CBD administration in the ration is valid for the taxon. Overall, since CBD did not reduce fish body weight gain and improve fish welfare state in the aforementioned parameters, CBD can be beneficial for both animals and fish farmers, who will maintain stable the amount of meat produced.

Although there was no CBD effect on growth variables, the 10 and 20 mg/kg doses increased fish's gonadosomatic index (GI). The GI is a macroscopic measure used to estimate the gonadal maturation in

several fish species[71,72]. The gonadal development can be impaired by high cortisol levels, decreasing the GI[73]. Thus, high GI values indicate good rearing conditions[74] and signalize fish welfare state. Nile tilapias treated with high doses of CBD (10 and 20 mg/kg) had lower baseline cortisol levels in the fifth experimental week. Although the cortisol level was not accessed in the first four experimental weeks, perhaps it was already low and thus enabled fish testes increased development. Additional research can elucidate how long it takes for CBD to decrease the baseline cortisol to confirm or reject this hypothesis and also may investigate the CBD effect on other important hormones for the gonadal development, such as sexual hormones (e.g., testosterone, progesterone, and estradiol)[75].

Although fish testes' size increased with CBD treatment, there was no increase in spermatozoa number. Fish treated with 1 and 10 mg/kg of CBD presented fewer spermatozoa than control fish. In particular, the 1 mg/kg dose drastically decreased the number of spermatozoa cells (Fig. 6a and b). Indeed, the endocannabinoid system plays a key role in regulating spermatogenesis[76]. The CB2 receptors are expressed in all the cell stages of spermatogenesis, and it was suggested that their activation by endocannabinoids, such as the anandamide, is related to the regulation of the spermatogenesis mitotic cells[77]. Meanwhile, another target receptor of anandamide, the transient receptor potential cation channel subfamily V member 1 receptors (TRPV1), are highly expressed in the meiotic stage and possibly are involved in the control of this spermatogenesis stage[77]. Depending on the dose CBD can (1) indirectly increase the anandamide supply and activate more CB1 or CB2 receptors, (2) or block the CB2 or CB1 receptors and/or activate the TRPV1 receptors (biphasic effect)[30,32]. Therefore, different drug doses can cause distinct disturbances on the spermatogenesis axis[30,77]. Our results showed that low doses of CBD substantially decreased the number of spermatozoa, and higher doses promoted a smaller decrease on it (Fig. 6). We did not analyze the effect of CBD on other stages of spermatogenesis, such as cells pre-meiotic. Maybe, smaller doses such as the 1 mg/kg dose led to an increase of pre-meiotic cells and a decrease in the post-meiotic cells, while higher doses could have the opposite effect. Further studies are necessary to unravel how the biphasic effect of CBD can regulate the entire spermatogenesis.

Other studies did not find CBD effect on the number of spermatozoa[78], but reported deregulation on spermatogenesis, impairment of sperm quality[30,78], motility[38,77,78], and also reduction in sperm fertility[77,79] in several animal species (sea urchins, CBD concentration of 0.1–10 μM[79]; rats, 15 and 30 mg/kg[30]; and zebrafish, CBD concentration of 0.5 μM[38]). However, there is no general agreement regarding the reversibility or not of the reproductive toxicity effects of CBD on the male reproductive system[30]. It seems that the period of exposure to the drug can lead to reversible or irreversible effects of CBD on the reproductive system[30]. Exposures during gonadal development can lead to irreversible and long-term effects[30,80], while exposures after this period lead to reversible effects[30,77]. Therefore, to promote CBD as a tool to improve animal welfare, these toxic effects on males' reproductive system must be considered. In addition, there must be a better comprehension of whether these effects are reversible or irreversible and at what age the use of CBD is safe for the male reproductive system.

Finally, CBD seems to be a promising tool for increasing fish welfare in captive environments since, depending on the dose (10 or 20 mg/kg), the drug decreases animals' aggressiveness (10 mg/kg), non-social stress (20 mg/kg) and baseline cortisol levels (10 and 20 mg/kg), besides increases testes size (10 and 20 mg/kg). CBD can easily be offered in fish diets, and the 20 mg/kg can be more suitable to improve the welfare of non-aggressive social fish species, while the 10 mg/kg dose can be used to improve the welfare of aggressive fish species, such as the Nile tilapia. However, it is necessary to be cautious when using the 10 mg/kg dose for Nile tilapias reared for reproduction purposes since it significantly reduces fish spermatozoa. We understand that this is the first time that evidence has been shown of CBD's potential as a tool to improve the welfare of captive and farmed animals. We highlight that CBD can improve fish welfare and it does not reduce fish growth. These results provide a win-win situation for both animals, which will have life quality, and fish farmers, who can keep their production while adding value to their product. Currently, CBD is not a cheap resource, but low concentrations, as used in this study, have already presented promising results. Besides, Cannabis is increasingly being socially accepted, and there is a trend for legalization in many countries. Thus the prices may decrease in the near future[64]. In this study, we used a laboratory approach, isolating fish to obtain the CBD's effect on animals' behavior, physiology, and morpho-histological aspects related to welfare. Thus, before implementing CBD in fish farms, further studies are necessary to test whether the welfare improvement observed in our study is maintained in aquaculture conditions, if CBD affects the fish meat quality and if there is an accumulation of the drug in the meat and water.

Methods

Animals and experimental conditions

Juveniles of Nile tilapia (Supreme strain and sexually reversed – all males) were obtained from fish farming at Botucatu – SP, Brazil, and used to constitute a stock population (500L tank, 100 fish). Afterward, 60 fish were selected for the experiment beginning and were individualized in 23L aquaria (40 x 23 x 25 cm) without visual contact. Aquaria's water ranged between 25–27°C, was supplied with constant aeration, and a 12-h light cycle (6:00 to 18:00 h) was set. Water was partially changed (40%) every two days to maintain quality parameters (pH: 6.8–7.2, ammonia ≤ 0.05 ppm, and nitrite ≤ 0.5 ppm). Fish were fed with a treated commercial diet (Presence Nutripiscis – extruded ration, pellets of 3-4mm) corresponding to 3% of their body weight fractionated at three meals in a day (9:00h, 13:00h, and 17:00h). At the beginning of the experiment, the average fish weight was 29.5 ± 2.8g (mean ± SD). All tests were conducted at 8:00–12:00h with fasted animals.

Cannabidiol and treated ration preparation

The CBD used in this study was isolated in salt (99% concentration) and was obtained in collaboration with the Universidade Federal de São Paulo (UNIFESP). CBD is a highly liposoluble drug[81]; therefore, to add it to the fish diet, the drug was diluted in soybean oil, and a top coating with this mix was applied to commercial pellets.

The CBD dose of each treatment (0 mg/kg, 1 mg/kg, 10 mg/kg, and 20 mg/kg) was calculated based on fish's initial weight (approximately 30g; Table 2) without adjustments through all experiment. The total CBD amount applied to each treated diet (Table 2) was calculated based on the following formula:

$$CBDamountappliedtothetreateddiets = CBDdose(mg) \times 15fish \times 35days$$

In which 15 is the N of fish per treatment and 35 days the experimental period

The treated rations preparation was conducted in a room without light due to CBD photosensitive property[82], and 2 kg of pellets were used for each treatment. The total CBD amount of each treated diet (Table 2) was dissolved in a quantity of soybean oil corresponding to 2% of 2 kg of ration. To dissolve CBD in soybean oil, a heat magnetic stirrer (Biomixer AM-10), at 60°C, rotating at 3000 rpm was utilized. Afterward, the pellets were placed homogeneously on a straight surface, and the oil with CBD was applied to it with a hand sprayer. To ensure that CBD was applied in all superficies, the pellets were turned four times during oil application, and after that, they were put in a plastic bag and shaken for 3 minutes. Lastly, the pellets were placed on a surface and left out at room temperature for one day to dry. The same procedure was done to prepare the control group diet, however, without CBD addition.

Mirror Test – Aggressive behavior and response to a social stimulus

The mirror test was used to assess fish's aggressive behavior and stress response to a social stimulus in the first four weeks (Fig. 1). The mirror test is a reliable method to evaluate fish aggressive behavior in several species, including the Nile tilapia[46,83]. Cichlid fishes (for example, the Nile tilapia) cannot recognize their image in the mirror, perceiving it as another conspecific entering their territory. Thus, they exhibit aggressive interactions against the mirror[84]. Moreover, fish reflected image in a mirror (social stimulus) induce stress responses in individuals[46].

In the mirror test, each fish was recorded for ten minutes (N = 15). On one side of the aquarium, a mirror of the same size as the lateral wall was placed parallel to this, covered by an opaque divisor. The recording started when the opaque divisor was removed. The latency for the fish to perform the first aggressive behavior against the mirror and the frequency of attacks against the mirror (bites, touches and lateral fights[53]) were accounted. Fish that not attack the mirror in any of the sample time points analyzed were excluded from the analysis.

Moreover, the VR pre- and post-social stimulus (mirror test) was measured (N = 15). The VR is a physiological response of fish and a reliable measure of stress level since it increases in response to stressors and is related to the metabolic rate[54]. To measure the VR, the time that fish took to perform 20 successive opercular or buccal movements was accounted[54]. Next, it was calculated how many opercular beats per minute each fish would execute through an estimate. Both VRs (pre- and post-social stimulus) were measured three times per fish in three consecutive minutes. The mean for each fish was calculated and used for further analysis[54].

Stress responses to a non-social stimulus - Confinement stress

In the experiment's fifth week, some fish's stress responses (specifically, cortisol level and VR) to non-social stress were measured. On the first day of the week (experimental day 29, Fig. 1), blood sampling for cortisol assay was performed (baseline levels). On the last day of the week (experimental day 35, Fig. 1), confinement stress (non-social stimulus) was applied to fish. To this end, an opaque partition was used to restrict fish to only 10% of the aquarium volume for 30 minutes. The VR pre- and post-confinement were measured (N = 14, since some fish died before the first blood sampling). Moreover, a second blood sampling for cortisol assay was performed (stress-induced levels) 30 min after the confinement[85].

Cortisol assay

Fish were taken off their aquaria with an aquarium dip net (N = 8), anaesthetized by immersion in an aquarium with clove oil (280 mg/l), and posteriorly a blood sample was collected (0.4ml) through cardiac punction with hypodermic needles and heparinized syringes. After this procedure, fish were returned to their aquaria. The handling time between removing and returning fish to the aquarium lasted less than 5 minutes for all individuals to avoid any bias of handling time in the cortisol levels. The blood was centrifuged at 3000 rpm for 10 minutes to separate approximately 0.1 ml of plasma, which was frozen at -20°C for further cortisol assays. The cortisol was measured by ELISA – Enzyme Linked Immunosorbent Assay, using commercial cortisol kits (DRG, Marburg, Germany).

Feed ingestion and growth variables

Daily, 39 pellets were offered to each fish (approximately 0.9 g - equivalent to 3% of animals' initial weight), divided into three meals (9:00h, 13:00h and 17:00h; 13 pellets per meal). After a meal, the pellets left in each aquarium were accounted for and removed right before the next meal. The daily feed ingestion was calculated by subtracting the number of pellets left of 39 (total offered in a day). Afterward, the consumed mass of feed in a day was inferred from this value. Lastly, the consumed mass of feed each week was calculated for further analysis.

At the end of the fourth week, fish were weighed, and the following growth variables were measured (N = 15): final standard length, final weight, average weight gain (AWG), feed conversion (FC), specific growth rate (SGR), and condition factor (K). The AWG, FC, SGR, and K were calculated through the following formulas, respectively:

$$AWG = Final weight(g) - Initial weight(g)$$

$$FC = \frac{Feed consumption(g)}{AWG}$$

$$SGR = \frac{\ln Final weight(g) - \ln Initial weight(g)}{Experimental period(days)} \times 100$$

$$K = \frac{FinalWeight(g)}{Finalstandardlengt(cm)^3} \times 100$$

Morpho-histological analysis of fish's reproductive system

At the end of the fifth experimental week, testes were collected for morphological and histological analyzes. Right after being collected, the gonads were weighed, and the gonadosomatic index (GI) (N = 10) was calculated flowing the formula:

$$GI = \frac{Gonadweight(g)}{Fishweight(g)} \times 100$$

Afterwards, the testicular explants were fixed in 4% Karnovsky at 4°C for at least 24h, dehydrated and embedded in a Technovit (7100) historesin (Heraeus Kulzer, Germany). Subsequently, the samples were sectioned at 3µm of thickness and stained with 0.1% toluidine blue. The histological sections obtained were used to quantify the relative number of spermatozoa of fish (N = 5). Twenty non-overlapping fields were randomly chosen and photographed using a Leica DMI6000 microscope (100x objective lens total magnification). The ImageJ software was used to account for the number of spermatozoa in each field[86,87]. The mean spermatozoa number by field was calculated for each fish for further analysis

Statistical analysis

All the statistical analyses were performed in the R environment software (v3.6.0.). Shapiro-Wilk and Levene tests were used to test the normality and homoscedasticity of data, respectively. For the stress response variables measured in the fifth week (VR pre- and post-confinement, ΔVR, cortisol baseline and stress-induced levels), growth variables (final standard length, final weight, AWG, FC, SGR and K) and morpho-histological variables of reproduction (GI and spermatozoa number by field), an ANOVA one-way was performed when the data met the parametric assumptions, and a Kruskal-Wallis test was done when data did not met any parametric assumption. All the response variables mentioned above were predicted by "treatment", a 4-level (levels: control, CBD 1, CBD 10, CBD 20) categorical independent variable. Post-hoc comparisons were performed using the Tukey HSD test.

Regarding the following response variables: aggressive behavior (number of attacks and latency for the first aggressive behavior), stresses responses to a social stimulus (VR pre- and post-social stimulus and ΔVR) and feed ingestion, LMM were performed. The independent categorical variable "treatment" (levels: control, CBD 1, CBD 10, CBD 20) and "sampling time points" (e.g., levels: basal, week 1, week 2, week 3 and week 4) were set as fixed factors in the models, and "fish ID" was included as a nested random factor. The normality of residuals assumption was checked through visual analysis of Normal quantile-quantile plots (QQ plots) of residuals (using the "qqnorm" function in R) and also the Shapiro-Wilk test. The assumption was valid for all mixed models built on this study. Post-hoc comparisons were performed using the Tukey HSD test. The significance level for all statistical tests performed in this work was set at $\alpha = 0.05$.

Ethical note

The current research was conducted in accordance with the Ethical Principles on Animal Experimentation adopted by the National Council for the Control of Animal Experimentation (CONCEA/Brazil). All procedures used in this study were approved by the CEUA (Committee on Ethics in the Use of Animals) of São Paulo State University (UNESP), protocol # 4166190321. The study was carried out in accordance with ARRIVE guidelines (https://arriveguidelines.org). The plant material used in this study complied with relevant institutional, national, and international guidelines and legislation.

Declarations

Data Availability Statement

The data that support the findings of this study are available in supplementary Excel data set files.

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Author contributions statement

Conceptualization: B.C-d-S., R.F. Study Design: B.C-d-S., M.S.B., I.I.G., J.F., P.C.G., R.F. Data collection: B.C-d-S., J.F., M.S.R., D.F.C., R.H.N. Data curation and Formal analysis: B.C-d-S. Figures: B.C-d-S, I.I.G. Writing: B.C-d-S., M.S.B., I.I.G., E.G., P.C.G., Supervision: P.C.G. All authors reviewed the manuscript.

Additional information

Competing interests

The author(s) declare no competing interests.

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Figures

Figure 1

Experimental timeline and design - sequence of events during all the experiment. The procedures start with fish acclimatizing to the isolated aquarium for 7 days (week). Followed this step, firstly, on day 1, basal fish's aggressiveness level (represented in the figure by the green "A" letter) and stress response [Ventilation rate (VR)] to a social stimulus (represented in the figure by the green "B" letter") were accessed through the mirror test. Afterward, fish started to receive the treated diets containing different cannabidiol (CBD) doses - 0 (control), 1 (CBD 1), 10 (CBD 10) and 20 mg/kg (CBD 20). During the first four experimental weeks (day 1 to day 28), the aggressive behavior and the stress responses to a social stimulus were accessed once a week. In the fifth experimental week, some stress responses to a non-social stimulus (confinement stress) were measured (represented in the figure by the yellow "C" letter): on day 29, blood samples were collected to analyze fish's cortisol baseline levels; and on day 35, confinement stress was applied to fish, and the VR and cortisol stress-induced levels were collected. Lastly, fish were euthanized (1500µl/l of clove oil), and their testes were collected for morpho-histological logical analysis (gonads' size and the number of spermatozoa).

Figure 2

Effect of diets containing different cannabidiol (CBD) doses, 0 (control), 1 (CBD 1), 10 (CBD 10) and 20 mg/kg (CBD 20) on (**a**) the number of attacks and (**b**) the latency for the first aggressive behavior of Nile tilapias (N = 15). These response variables were evaluated over five sampling time points, basal, week 1, 2, 3 and 4. The arrow indicates the beginning of the application of the treated diets. The graphs on the

left present the mean values without SEM of all CBD treatments over the five sampling time points, while the graphs on the right present the mean ± SEM values of only the treatments that presented significant differences over the sampling time points. The asterisks above a mean value indicate significant differences between this measurement and the baseline measurement of the same groups (Tukey HSD test, $p < 0.05$).

Figure 3

Effect of diets containing different cannabidiol (CBD) doses, 0 (control), 1 (CBD 1), 10 (CBD 10) and 20 mg/kg (CBD 20) on (**a**) the ventilation rate (VR) pre-social stimulus, and (**b**) the VR post-social stimulus of Nile tilapias (N = 15). The social stimulus applied to fish was the mirror test, in which the fish's image reflected in the mirror was used to induce stress responses in animals. The response variables were evaluated over five sampling time points, basal, week 1 (W1), 2 (W2), 3 (W3), and 4 (W4). The arrow indicates the beginning of the application of the treated diets. The graphs on the left present the mean values without SEM of all CBD treatments over the five sampling time points, while the right graphs present the mean ± SEM values of only the treatments that presented significant differences over the sampling time points. '#' above a mean value indicates a significant difference between this measurement and the control and CBD 1 measurements at the same sampling time point (Tukey HSD test, $p < 0.05$). '*' above a mean value indicates significant differences between this measurement and the baseline measurement of the same treatment (Tukey HSD test, $p < 0.05$).

Figure 4

Effect of diets containing different cannabidiol (CBD) doses, 0 (control), 1 (CBD 1), 10 (CBD 10) and 20 mg/kg (CBD 20) on the ΔVR (VR post – VR pre-confinement) of Nile tilapias (N = 14). The stress applied was a confinement stressor (non-social stress). The highlighted p-value indicates a significant difference between treatments under the Tukey HSD test ($p <0.05$). Values are mean ± SEM).

Figure 5

Effect of diets containing different cannabidiol (CBD) doses, 0 (control), 1 (CBD 1), 10 (CBD 10) and 20 mg/kg (CBD 20) on the baseline plasma cortisol levels of Nile tilapias (N = 8). Fish received the treated diets for 28 days before the baseline measure was collected. Highlighted p-values indicate a significant difference between treatments under the Tukey HSD test ($p <0.05$). Values are mean ± SEM.

Figure 6

Ex vivo effects of 0 (control), 1 (CBD 1), 10 (CBD 10) and 20 mg/kg (CBD 20) of cannabidiol (CBD) on (**a**) gonadosomatic index (GI) of Nile tilapias (N = 10) and (**b**) the mean spermatozoa (SPZ) number by field (total of 20 fields by fish) in testicular explant from Nile tilapias (N = 5). Fish were exposed for 35 days to the CBD. Different uppercase letters indicate significant differences between treatments (Tukey HSD test, $p < 0.05$). The number of SPZs was determined by morpho-histological analysis using ImageJ software. Values are mean ± SEM. (**c**, **d**, **e**, **f**) Histological sections of Nile tilapia testes following 35-days of exposure to CBD. Spermatozoa areas are indicated by SPZ in each figure (scale: 50 µm).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- DatasetFeedIngestion.xlsx
- DatasetFigure2Aggressivebehavior.xlsx
- DatasetFigure3Stressresponsetoasocialstimulus.xlsx
- DatasetFigure4Ventilationrateresponsetoanonsocialstimulusconfinement.xlsx
- DatasetFigure5Cortisolbaselineandstressinducedlevels.xlsx
- DatasetFigure6aGonadosomaticindex.xlsx
- DatasetFigure6bSpermatozoanumber.xlsx
- DatasetTable1Growthvariables.xlsx