Short communication

Solfeggio-frequency music exposure reverses cognitive and endocrine deficits evoked by a 24-h light exposure in adult zebrafish

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ABSTRACT

Music therapy has long been used as a non-pharmacological intervention to improve cognitive function and mood in humans. Mounting rodent evidence also supports beneficial impact of music exposure on animal cognitive performance. The zebrafish (*Danio rerio*) is an important emerging aquatic animal model in translational biomedical and neuroscience research. Here, we evaluate the effects of intermittent (2-h or 6-h twice daily) and continuous (24-h) solfeggio-frequency music exposure on behavioral, cognitive and endocrine parameters in adult zebrafish whose circadian rhythm was disturbed by a 24-h light exposure. Overall, a 24-h light exposure stress evokes overt cognitive deficits in the inhibitory avoidance test and elevates zebrafish whole-body cortisol levels. However, these effects were reversed by solfeggio-frequency music exposure for 2 or 6 h twice daily, and by continuous 24-h exposure. Collectively, these findings suggest a positive modulation of cognitive and endocrine responses in adult zebrafish by environmental enrichment via the long-term exposure to music, and reinforces zebrafish as a robust, sensitive model organism for neurocognitive and neuroendocrine research.

Music therapy has long been used to improve cognitive function in humans. For example, patients with Alzheimer disease (AD) show improved categorical word fluency [1], autobiographical memory [2] and memory for lyrics [3] after listening to music. Music, including using its solfeggio frequencies, also reduces stress in students [4], whereas music therapy with singing training improves psychomotor speed in AD patients [5]. Patients with AD and other neurodegenerative diseases frequently present circadian rhythm disturbances, which can be associated with cognitive deficits [6] and corrected by music therapy [7]. Paralleling clinical data, experimental studies in healthy rodents and in rodent central nervous system (CNS) disease models reveal similar beneficial effects of music [8], such as improved task acquisition, as well as working [9] and spatial memory [10].

In addition to promotive human cognitive performance, music therapy can also improve the quality of sleep [11,12] in both older adults [13] and students [14]. However, while music therapy beneficially affects sleep and cognition, its effects on circadian rhythms and neuroendocrine endpoints have not been examined in-depth [15]. Animal models are a valuable tool for probing behavioral and endocrine effects of environmental enrichment, incuding music exposure [8] which exerts robust memory-enhancing effects in rodent models (e.g., Morris water maze test for spatial cognition) [16].

With the growing utility of zebrafish (*Danio rerio*) in biomedicine [17–19], this aquatic model organism may complement traditional

rodent models in translational neuroscience research. Indeed, in addition to high genetic and physiological homology to humans [20], zebrafish also possess generally conserved neurotransmitter systems, neural circuits and neuromorphology [21,22]. Paralleling rodent evidence, zebrafish have also demonstrated some effects of music exposure on brain and behavior [23]. For example, adult zebrafish exposed for 2 h to Vivaldi music at 65–75 dB twice daily for 15 days, demonstrate reduced anxiety-like behavior in the novel tank test and the light-dark test, and lower peripheral levels of pro-inflammatory cytokines, such as interferon gamma (INF-γ) [24].

Collectively, this raises the possibility of using zebrafish to further probe the effect of music exposure on neurobehavioral processeses and their potential interplay with circadian rhythms and cognitive performance. Addressing this problem, here we examine the effects of music exposure on adult zebrafish cognitive performance and whole-body cortisol levels following a 24-h light exposure stress, chosen here as a well-established disruptor of normal behavior and circadian rhythms in zebrafish [25].

Briefly, a total of 192 mature adult zebrafish (at ~1/1 male/female ratio) of the wild-type outbred short-fin strain were obtained from a commercial supplier (Delphys, Porto Alegre, Brazil) and housed 2 fish/L in 50-L tanks equipped with biological filters, under constant aeration and a photoperiod (14-h light/10-h dark cycle). Water temperature was maintained at 27 ± 0.5 °C; pH 7.0 ± 0.2, with dissolved oxygen kept at

5.9 ± 0.15 mg/L, total ammonia at < 0.01 mg/L, total hardness at 6 mg/L, and alkalinity at 22 mg/L $CaCO_3$. Animal experimentation reported here was approved by the Institutional Animal Care Committee and fully adhered to National and International guidelines on animal experimentation, and the accepted principles of ethical experimentation. The outbred population selection for the present study was based on population validity considerations and their relevance for the present study. Briefly, although genetically controlled models (e.g., inbred zebrafish strains) can be more reproducible and reliable system for neurogenetics research, modeling CNS disorders, such as in the present study, involves 'real' human disorders affecting genetically heterogeneous populations [26]. Thus, using outbred zebrafish strain (such as selected here) was deemed a more populationally valid and translationally relevant approach for the purpose of this study.

The present study assessed the effects of intermittent (2-h or 6-h twice daily) or continuous (24-h solfeggio-frequency) music sessions on zebrafish cognitive responses vs. unexposed controls, using 24 animals per group in 4 music exposure groups (non-exposure, 2-h or 6-h twice daily, and continuous 24-h music exposure) with two circadian cycle groups (14-h light/10-h dark cycle and 24-h light exposure). The fish cognitive retention index was assessed in the inhibitory avoidance test in animals subjected to normal circadian cycle (14-h light/10-h dark cycle) and 24-h light exposure, chosen here as an established sleep deprivation and cognitive deficit models in zebrafish [25,27]. The
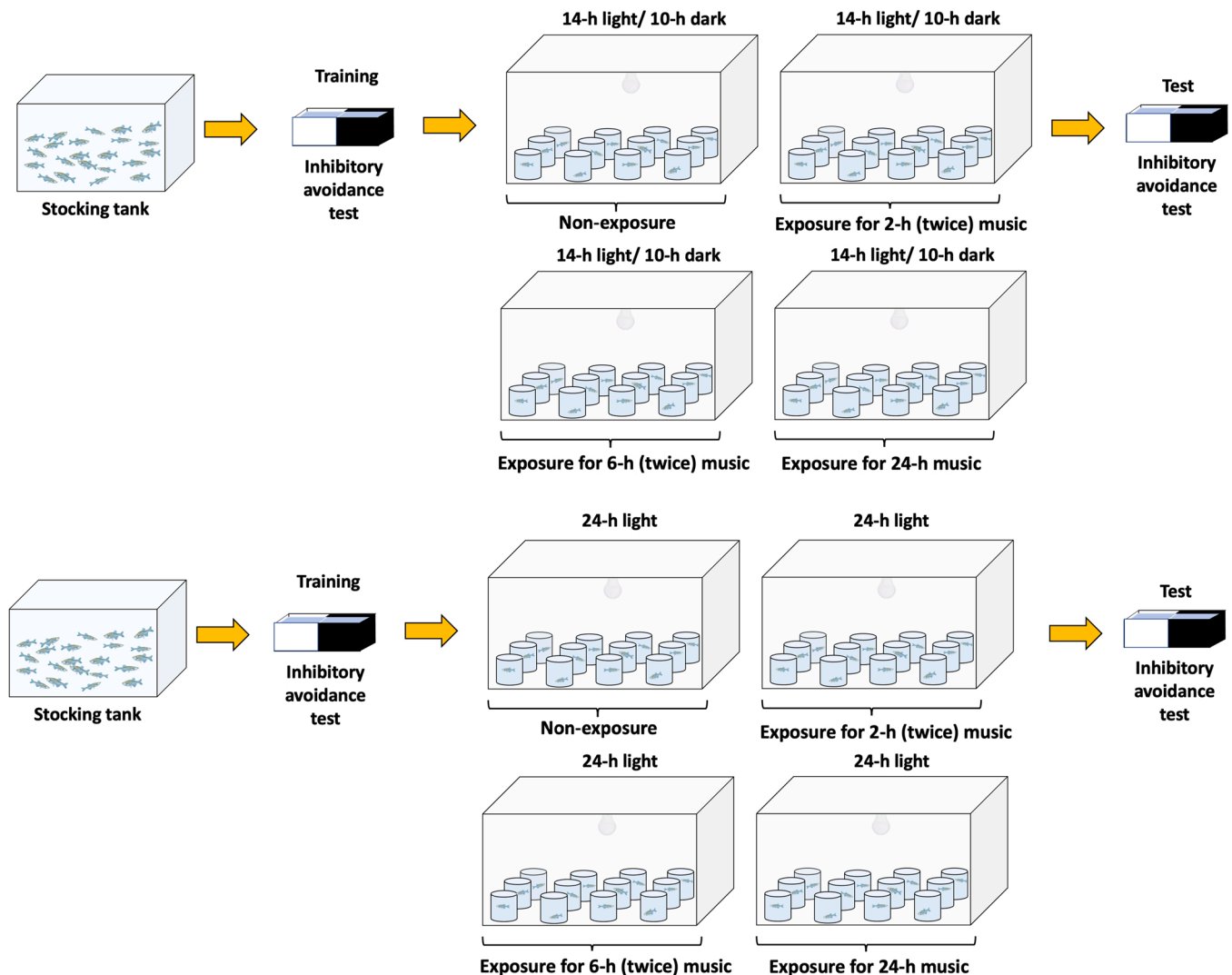

Fig. 1. Schematic diagram illustrating the study experimental design.

inhibitory avoidance test is based on the innate response to enter a dark (black) and to avoid a brightly-lit (white) environment, and an avoidance of a shock delivered in the black compartment, already demonstrated as a robust test of zebrafish cognition [18].

The training in the inhibitory avoidance test was performed prior to the light cycle and music treatments. After the training test, fish were kept in isolated tank, with only visual contact with conspecifics, as in [25]. In their respective tanks, fish were kept under either normal or disturbed (24-h light) cycles, as well as exposed to music, in four tanks placed in four large boxes (65 width × 90 length × 65 height cm) made of a wooden frame with light beige canvas as walls, floor and ceiling, each with one ceiling-mounted light bulb (4.9 W) as in [25]. From inside, the boxes were coated with two layers of foam and egg crate paper. The fish were subjected to two music sessions (2-h or 6-h) or to 24-h continuous music [28]. The study experimental design is summarized in Fig. 1.

The music sessions were performed using the solfeggio frequency of 963 Hz (available online at www.youtube.com/watch?v=UkgSbK_PjXw). In humans, solfeggio frequencies have been used for studying musical perception and learning [29,30], as well as to explore putative therapeutic effects of music on emotions using the artificial intelligence (e.g., machine learning) model [31]. Music and background noise intensity and frequency in the present study were assessed outside the water from a distance of 60 cm from the tanks, using a smartphone Sound Level Meter application (www.play.google.com/store/apps/details?id=com.bolshakovdenis.soundanalyzer). The music loudness level was 50–70 dB, with the ambient noise level inside the box of 20 dB.

Zebrafish cognitive phenotypes were assessed in a rectangular glass apparatus (18 length × 9 width × 9 height cm), individually recording fish for 5 min using an HD C525 web-camera (Logitech, Inc., Romanel-sur-Morges, Switzerland). The videos were manually analyzed, calculating the latency to enter the black (shock) compartment, and assessing cognitive retention (computed as the difference between the latency to enter the black compartment during the training vs. testing sessions, as in [32]). The inhibitory avoidance test was performed to assess zebrafish cognitive function, according to [32]. Briefly, the rectangular tank was divided into two equal black and white compartments, separated by a manually operated opaque guillotine-type partition (Fig. 1).

The black chamber contained two copper plates one on each side, connected to a 12-V electric generator producing a 100-mA, 3.2 ± 0.2 V AC current at 100 Hz between the copper plate and the center of the black compartment. During the training session, zebrafish were individually placed on the white side of the tank. After a 1-min acclimation, the guillotine door was lifted, allowing zebrafish to enter the black compartment (assessed by the mid-body point crossing the line between the compartments). When fish crossed into the black side of the tank, the sliding partition was closed and a mild electric shock was administered for 5 s using a 12-V electric generator. All fish were individually exposed to the training sessions (only one training session per fish was used in the present study), and then gently removed from the test apparatus and placed in their respective hometanks (1 fish per tank) for acclimation (Fig. 1).

Memory retention was assessed in the same fish 24 h later, similarly to the training session. The cognitive retention index was defined as the difference in the latency to enter the black compartment of the tank between the training vs. testing sessions, as in [32]. In general, the fast entering the black compartment was deemed a cognitive impairment in the inhibitory avoidance test, according to [32].

At the end of behavioral tests, a total of 15 fish from each group were euthanized in ice-cold water, followed by decapitation (after the cessation of opercular movements), and stored at − 20 °C for further whole-body cortisol analyses. To assess their cortisol levels, the animals were weighted and pooled 3 bodies per sample (n = 5 per group) and homogenized using a Tecnal tissue homogenizer (Piracicaba, Brazil) in 3 mL of phosphate buffered saline for 1 min, prior to whole-body cortisol extraction with diethyl ether, as described previously in [33].

Cortisol levels were analyzed using a commercially available cortisol enzyme-linked immunosorbent assay (ELISA) kit from Diagnostic Biochem Canada Inc. (London, Canada). The detection accuracy was tested by calculating the recoveries from standard samples containing 50, 25 and 12.5 ng/mL cortisol), with the mean detection of 90%. All cortisol values were adjusted for recovery (cortisol value = measured value × 1.1) and normalized based on the weight of the respective whole-body samples, expressed as absolute cortisol concentrations (ng/g body weight).

The data normality and homogeneity of variance were assessed by the Kolmogorov-Smirnov and the Levene tests, respectively. The difference between the training and the testing sessions' latency to enter the black (shock) compartment in each group was compared using the Wilcoxon matched-pairs test, since data were collected from the same individual fish single-housed in their respective hometanks (Fig. 1). The cognitive retention indices between the music treatment groups (unexposed controls and 2-h or 6-h (twice daily) or 24-h music exposure) were compared using the Kruskal-Wallis test. The effect size was calculated using Eta squared (η^2) for Kruskal-Wallis test comparisons. Whole-body cortisol data were normally distributed and therefore compared using the two-way ANOVA test (factors: music treatment and light cycle), followed by the Tukey post-hoc test for significant ANOVA data. P was set as < 0.05 in all analyses. All data were analyzed using GraphPad Prism 8.2.1 statistical software (GraphPad Software, San Diego, USA). The study experimental design and its description here, as well as data analysis and presenting, adhered to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines for reporting animal research and the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for planning animal research and testing [34,35].

Under normal 14-h light/10-h dark cycle, both unexposed controls (p = 0.0001) and fish exposed to 2-h music (p = 0.0218) showed increased latency to enter the black (shock) compartment during the test, compared to their respective initial training, sessions (Fig. 2A). In contrast, fish exposed to 6-h (p = 0.966) and 24-h (p = 0.3781) music sessions did not differ in the latency to enter the black (shock) compartment during the test, compared to their respective initial training sessions (Fig. 2A).

Under disturbed 24-h light cycle, unexposed control zebrafish shortened the latency to enter the black (shock) compartment during the test, compared to their respective initial training sessions (p < 0.0001, Fig. 2B). In contrast, zebrafish exposed to music for 2-h (p = 0.0131), 6-h (p = 0.0003) or 24-h (p = 0.0325) all increased the latency to enter the shock compartment during the test, compared to their respective initial training, sessions (Fig. 2B).

In addition, music exposure for 2-h, 6-h and 24-h evoked higher cognitive retention index in fish housed under a 24-light cycle (KW = 24.8, p < 0.0001, η^2 = 0.25 vs. control, Fig. 2 C), but not in fish housed under a 14-h light/10-h dark cycle (KW = 8.4, p = 0.0384, η^2 = 0.06 vs. control, Fig. 2C). Overall, these data confirm overt cognitive deficits evoked by 24-h light cycle exposure, whereas music exposure improved cognitive retention index in these fish. Finally, the 24-h light exposure elevated whole-body cortisol levels (interaction (F $_{3,32}$ = 12.4; p < 0.0001); light cycle (F $_{1,32}$ = 5.105; p = 0.0308) compared to all 14-h light/10-h dark cycle exposure groups, whereas the 2-h, 6-h, and 24-h music exposure reverted this effect (music treatment (F $_{3,32}$ = 15.6; p < 0.0001, Fig. 3).

In general, the present study demonstrated that music exposure improves cognitive performance in fish and normalizes their whole-body cortisol levels disturbed by 24-h light exposure (Figs. 2 and 3). These findings corroborate previous reports on cognitive and neuroendocrine (cortisol) deficits in zebrafish caused by 24-h light exposure [25], but are also in line with mouse data that constant light impairs animal cognitive behavior [36]. Furthermore, our fish results tend to parallel some recent human data, since the executive function declines in older men exposed to 24-h light [37]. Taken together, these findings
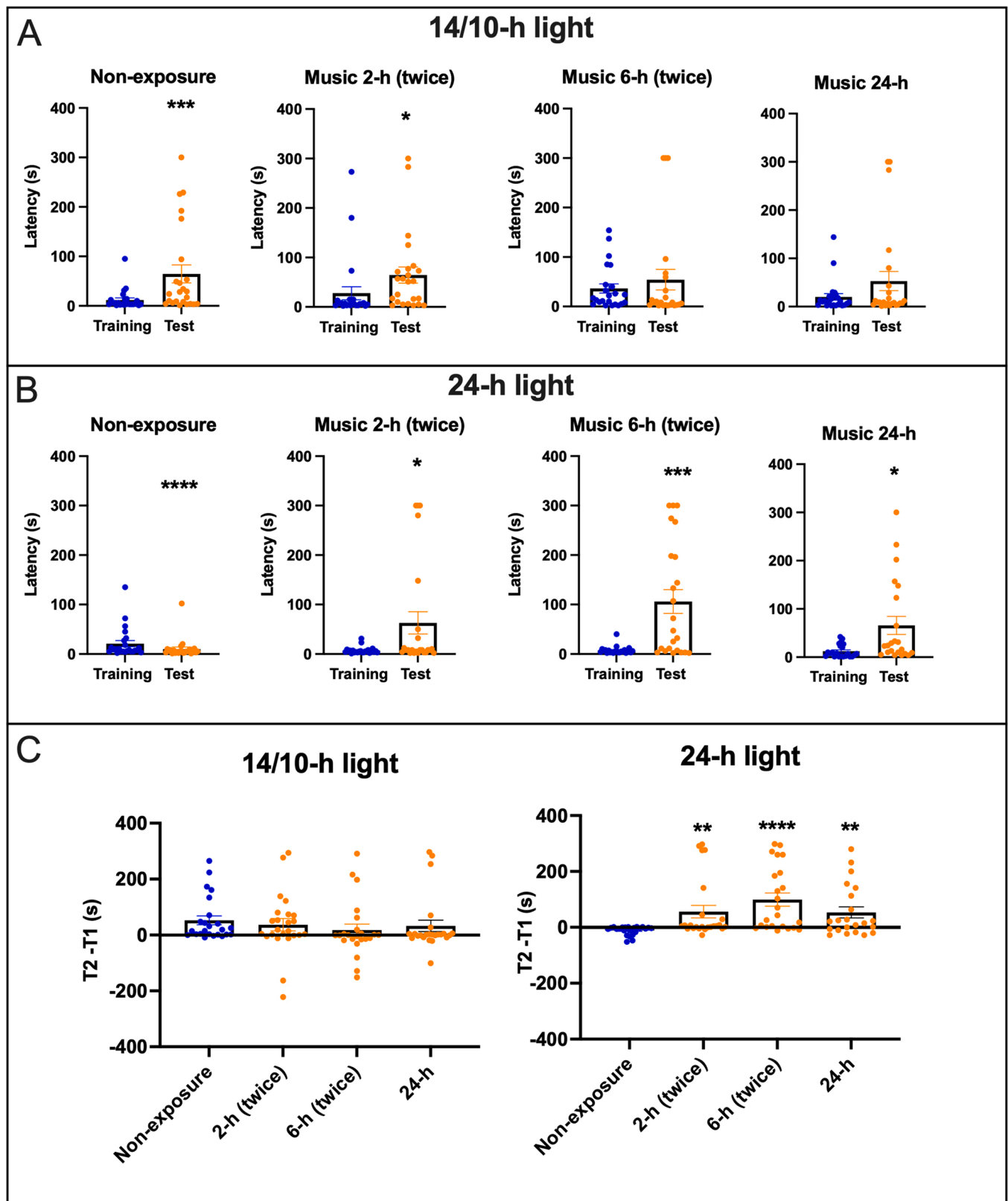
Fig. 2. Effects of music exposure on adult zebrafish cognitive performance in the inhibitory avoidance test. Data are expressed as mean $\pm$ SEM, *p $<$ 0.05, **p $<$ 0.01, ***p $<$ 0.001, ****p $<$ 0.0001, Wilcoxon matched-pairs test (latency time) or Kruskal-Wallis test (retention index data; n $=$ 24 per group). Effects of normal (A, 14-h light/10-h dark) and disturbed (B, 24-h light) circadian cycle and music exposure on adult zebrafish cognitive performance in the shock avoidance assay. (C) Cognitive retention index was calculated here as the difference between the testing latency time (T2) and the respective training latency time (T1) to enter the black (shock) compartment of the apparatus.
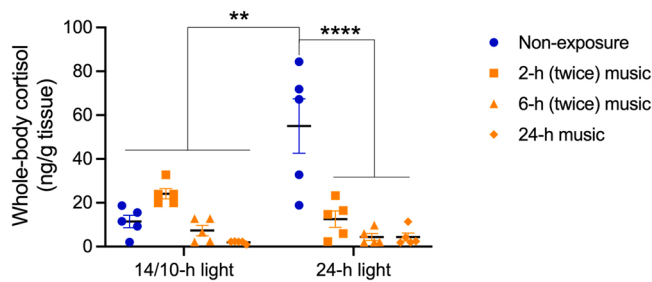
Fig. 3. Effects of the light cycle and music exposure on whole-body cortisol of adult zebrafish. Data are expressed as mean $\pm$ SEM, $**p < 0.01$, $****p < 0.0001$, two-way ANOVA test (factors: light cycle and music exposure), followed by Tukey pos-hoc test for significant ANOVA data (n = 5 per group, 3 fish pooled per sample). Interaction ($F_{3,32} = 12.4$; $p < 0.0001$); light cycle ($F_{1,32} = 5.105$; $p = 0.0308$); and music treatment ($F_{3,32} = 15.6$; $p < 0.0001$).

suggest shared, evolutionary conserved effects of acute light stress on cognitive and neuroendocrine responses.

In contrast, acute music exposure in the present study improved zebrafish cognitive performance (Fig. 3), paralleling rat data on better spatial memory following exposure to classical Mozart vs. minimalistic classical Glass music [38]. Furthermore, music exposure improves spatial cognition by enhancing the brain derived neurotrophic factor levels in dorsal hippocampus of developing rats [16], whereas acute music exposure enhances global efficiency of the brain, local neural efficiency in the prefrontal lobe, and sustained attention in humans [39]. In humans, solfeggio-frequency music also improves students musical memory capacity [39].

Notably, we found here that acute music exposure reverts increased cortisol levels induced by 24-h light exposure (Fig. 3), hence strikingly resembling corticosteroid-reducing effects of acute music in rodents [40] and humans [41,42]. For example, rats exposed to Mozart's Sonata K.448 display reduced serum corticosterone levels [43], and humans exposed to classical music during surgery show lower cortisol levels vs. unexposed controls [44]. Positive effects of music-based environmental enrichment have also been noted in zebrafish models as well [23]. For instance, zebrafish exposed to 2-h of Vivaldi music (65–75 dB) twice daily for 15 days display reduced anxiety-like behavior [24], and lower anxiogenic-like effects caused by 24-h social isolation [28]. Collectively, these and the present findings support music exposure as a positive modulator of stress-related neuroendocrine responses across taxons.

Clearly, there are some limitations of the present study. For example, here we used the same strain (wild-type outbred short-fin fish), mixed-sex (~1/1 male/female ratio) population and a single age (6 month) of zebrafish. Thus, the present study did not assess individual-, sex-, strain- or age differences in zebrafish cognitive responses to music and light exposure (which may affect the translatability into human phenotypes). In addition, we also tested only one type of music, and different music frequencies and exposure lengths can be expected to cause distinct CNS effects on animal behavior. For example, neuroplastic mechanisms may mediate improved cognitive functioning associated with long-term exposure to music, and these mechanisms may differ in musicians compared to non-musicians [45].

Furthermore, we also did not test brain or blood melatonin levels, to probe whether the light exposure for 24 h light modulates the levels of this hormone (and whether music exposure may rescue them), similarly to its effects on cortisol levels reported here (Fig. 3). Finally, the study only applied one assay, the inhibitory avoidance test, to evaluate zebrafish cognitive phenotypes. However, we fully recognize that a more comprehensive evaluation of fish performance in a battery of different cognitive tests (e.g, including both spatial and visual discrimination tasks, such as T-maze) in future studies may provide higher generality of our present findings.

In conclusion, the present zebrafish study generally corroborates

rodent and clinical data on pro-cognitive effects of music-based environmental enrichment [46,47] and their link to circadian rhythms [15]. Our findings suggest an evolutionarily conserved, positive modulation of cognitive and endocrine processes in zebrafish and mammals following solfeggio-frequency music exposure. This study also reinforces zebrafish as a robust, sensitive model organism for neurocognitive and neuroendocrine research, and calls for further use of zebrafish models for studying how cognitive and behavioral processes interact with circadian rhythms.

CRediT authorship contribution statement

Amanda C. dos Santos: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Murilo S. de Abreu:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Gabriel P. de Mello:** Investigation, Writing – original draft, Writing – review & editing. **Vanusa Costella:** Investigation, Writing – original draft, Writing – review & editing. **Nicoli R. do Amaral:** Investigation, Writing – original draft, Writing – review & editing. **Alexander Zanella:** Investigation, Writing – original draft, Writing – review & editing. **Júlia Poletto:** Investigation, Writing – original draft, Writing – review & editing. **Elena V. Petersen:** Writing – original draft, Writing – review & editing. **Allan V. Kalueff:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Ana C.V.V. Giacomini:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing.

Conflict of interest

The authors declare no conflicts of interest.

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