Title: Conserved serotonergic background of experience-dependent challenge-responding in zebrafish (*Danio rerio*).

Abbreviated title: Serotonergic background of challenge-responding in zebrafish

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Number of pages: 33

Number of figures: 6

Number of tables: 5

Number of words for abstract: 242

Number of words for introduction: 617

Number of words for discussion: 1487

Conflict of interest statement: The authors declare no competing interest.

Acknowledgement: We thank Christina Miskolczi (M.Sc., IEM, HAS, Budapest, Hungary) for language editing and Máté Tóth (Ph.D., IEM, HAS, Budapest, Hungary) for sharing comments that greatly improved the study. We also thank László Barna and Csaba Pongor, the Nikon Microscopy Center at the Institute of Experimental Medicine, Nikon Austria GmbH, and Auro-Science Consulting, Ltd., for kindly providing microscopy support.

Abstract

Forming effective responses to threatening stimuli requires the adequate and coordinated emergence of stress-related internal states. Such ability depends on early-life experiences and, in connection, the adequate formation of neuromodulatory systems, particularly serotonergic signaling. Here, we assess the serotonergic background of experience-dependent behavioral responsiveness employing a zebrafish (*Danio rerio*) model. For the first time, we have characterized a period during the behavioral metamorphosis in which zebrafish are highly reactive to their environment. Absence of social stimuli during this phase established by isolated rearing fundamentally altered the behavioral phenotype of post-metamorphic zebrafish in a challenge-specific manner, partially due to a decline in responsiveness and an inability to develop stress-associated arousal state. In line with this, isolation differently affected whole-brain 5-HT signaling in resting and stress-induced conditions, an effect that was present at the level of the dorsal pallium and was negatively associated with responsiveness. Administration of the 5HT1AR partial agonist buspirone prevented the isolation-induced serotonin response to novelty in the forebrain and rescued stress-induced arousal along with challenge-induced behaviors, which altogether indicates a functional connection between these changes. In summary, there is a consistent negative association between behavioral responsiveness and serotonergic signaling in zebrafish, which is well recognizable through the modifying effects of developmental perturbation and pharmacological manipulations as well. Our results imply a conserved serotonergic mechanism that context-dependently modulates environmental reactivity and is highly sensitive to experiences acquired during a specific early-life time-window, a phenomenon that was previously only suggested in mammals.

Significance statement

The ability to respond to challenges is a fundamental factor in survival. We show that zebrafish that lack appropriate social stimuli in a sensitive developmental period show exacerbated alertness in non-stressful conditions while failing to react adequately to stressors. This shift is reflected inversely by central serotonergic signaling, a system that is implicated in numerous mental disorders in humans. Serotonergic changes in brain regions modulating responsivity and behavioral impairment were both prevented by the pharmacological blockade of serotonergic function. These results imply a serotonergic mechanism in zebrafish that transmits early-life experiences to the later phenotype by shaping stress-dependent behavioral reactivity, a phenomenon that was previously only suggested in mammals. Zebrafish provide new insights into early-life-dependent neuromodulation of behavioral stress-responses.

Introduction

Coping with environmental threats is essential for the survival and well-being of animals. Contextually adequate emergence and expression of behavioral responses depend on previous experiences, particularly stimuli acquired during sensitive and plastic developmental periods (Chapman et al., 2004; Haller et al., 2014; Nederhof and Schmidt, 2012; Santarelli et al., 2014). While there are numerous plausible explanations regarding the function of this link, e.g. preparing the individual for later environmental challenges, the underlying mechanisms are poorly understood. Clinical and preclinical data suggest that the quality of development of neuromodulatory pathways, particularly the formation of the serotonergic system, affects later behavioral phenotype, hence it transmits the information of the early-life environment to adulthood (Booij et al., 2015; Fone and Porkess, 2008; Gross et al., 2002; Lehmann et al., 2003; Lukkes et al., 2009b; Raleigh et al., 1984). The diverse and context-dependent behavioral effects of serotonin suggest that these pathways, apart from direct modulation, are

able to act as general modulators of behavior (Geyer, 1995; Jing et al., 2009; Lovett-Barron et al., 2017; Yokogawa et al., 2012), possibly through the modification of internal states, such as arousal and alertness. State alertness adjusts behavioral reactivity to current environmental demands, e.g. the presence of acute stressors, by fine-tuning sensory-motor functions, information processing or decision-making processes. Despite its fundamental impact on the expression of behavior, there are only a few studies assessing the neuromodulatory basis of general arousal in healthy animals, while the literature of experience-dependent alertness and associated challenge-responding is even sparser.

The zebrafish (*Danio rerio*) provides an attractive tool to investigate basic vertebrate neurobiology, due to the ease and accessibility of approaches to modify or screen physiological processes (Lieschke and Currie, 2007; MacRae and Peterson, 2015). Particularly, the employment of zebrafish has several advantages to study the neural basis of experience-dependent challenge-responding: firstly, their relatively simple behavioral repertoire is primarily driven by robust stress responses including hormonal and neuromodulatory actions (Egan and Kalueff, 2009); secondly, their central nervous system possesses homologous anatomical structures and neurotransmitters to that of mammals with highly conserved function but less complex organization (Panula et al., 2010); thirdly, they show significant behavioral plasticity indicated by a robust behavioral reorganization occurring during the late larval stage (Lau and Guo, 2011). The onset of such metamorphosis gives us the opportunity to assess the impact of experiences in potentially sensitive developmental periods on the adult challenge-responding phenotype.

In the current study we aimed to utilize the above described unique features of zebrafish and assess how early-life events affect later challenge-responding phenotypes through alterations of 5-HT signaling. First, we aimed to identify a sensitive developmental period that plays a prominent role in shaping adult behavior. We hypothesized that the behavioral metamorphosis

at the late larval stage is characterized by environmental reactivity that may serve as a critical period for shaping the individual's later behavioural phenotype. Identifying such a sensitive period, we subsequently subjected zebrafish to the acute presence and/or chronic absence of environmental stimuli, e.g. social isolation and analyzed different aspects of challenge responsiveness. In order to do this, we conducted tests that represent non-meaningful stimuli i.e. an unidentified threat, such as environmental novelty and meaningful stimuli, such as a social cue. These contexts evoke different internal states and behavioral responses that may be orchestrated by different neuromodulatory actions (reviewed by Ralf-Peter Behrendt, 2011). We also differentiated between novel visual, social visual and novel but non-visual stimuli in order to exclude potential biasing effects of social isolation on sensory perception. Finally, we assessed isolation-induced changes in serotonergic signaling under resting and challenge-induced conditions by applying molecular, histological and pharmacological tools. We conducted measurements at whole-brain level and more specifically in limbic forebrain areas, then functionally correlated serotonergic and behavioral effects by the pharmacological blockade of 5-HT signaling.

Materials and Methods

Animals

Wild type (AB) zebrafish lines were maintained in the animal facility of Eötvös Loránd University according to standard protocols (Westerfield, 2000). Experimental subjects were unsexed animals aged between 8 and 30 dpf (days post fertilization). Fish were maintained in a standard 14 h/10 h light/dark cycle. Animals were terminated on ice immediately after each experiment. Feeding of larvae started at 5 dpf with commercially available dry food (a 1:1 combination of < 100 µm and 100–200 µm Zebrafeed, Sparos) combined with *Paramecium*. After 15 dpf, juvenile fish were fed using dry food with gradually increasing particle size (200–400 µm Zebrafeed, Sparos) combined with fresh brine shrimp hatched in the facility. All

protocols employed in our study were approved by the Hungarian National Food Chain Safety Office (Permits Number: PEI/001/1458-10/2015 and PE/EA/2483-6/2016).

Environmental modifications

Chronic social isolation (SI) of zebrafish was carried out between 14 and 29 dpf. Isolated animals were kept in white opaque plastic tanks (52 x 35 x 46 mm, depth x width x length) (Figure 2a) depriving the individuals of sensory cues from conspecifics. Tanks were filled with E3 medium, half of the volume of which was replaced on a weekly basis. Socially reared control animals were subjected to similar conditions with the exception of the size of their aquarium, which was matched to the greater number of larvae, providing approximately 50 ml volume for each larva.

Light/dark tank (LDT) exposure, considered as an acute challenge, was used as a developmental experience as well as a behavioral sampling tool. The procedure was identical in both cases and is described in the *Behavioral tests and analysis* section.

Drug treatments

Buspirone (Sigma-Aldrich) was dissolved in E3 medium and administered as a water bath for 10 minutes followed by a 1-min washout in compartments of a 24-well plate. Each compartment (15.6 mm in diameter) contained 1.5 ml of treatment solution. We applied 0 (vehicle) and 50 mg/l concentrations which were selected based on our previous studies (Varga et al., 2018).

Behavioral tests and analysis

For light/dark tank (LDT) testing, half of the base of a plastic tank (90 x 25 x 24, length x width x depth) (Fig1a) was masked with opaque matte black paint (dark zone), while the other half remained intact, penetrable by light (light zone). Experiments were conducted during the

second part of the light phase of the daily cycle, as zebrafish are reported to show continuous activity in this period (MacPhail, 2009). 8 subjects were tested simultaneously over the span of a single trial. Experiments in which pharmacological treatments were employed began with a 10-minute treatment bath in a 24-well plate compartment after which animals were gently pipetted to another compartment containing E3 medium for a 1-minute washout and then were moved to the testing apparatus for 10 minutes. Behavior of fish was recorded by a digital video camera. Animals were placed in the light zone of the apparatus directly next to the border of zones. Experimental tanks (Figure 1b) were each filled with 10 ml medium and thoroughly cleaned between trials. To reduce interfering stimuli from the environment, experimental apparatuses were covered with an opaque black plastic box with a hole on top allowing the attachment of the video camera. The apparatuses were illuminated from beneath with white LED panels covered by matte Plexiglas. Video recordings were analyzed using EthoVision XT 12 automated tracker software (NOLDUS L.P.J.J. et al., 2001) and Solomon Coder (András Péter, 2017) manual tracker software. Time spent in and transitions between light and dark zones were measured. Distance swum and mean velocity were measured only in the light zone. To describe scotophobia (dark avoidance) and scototaxis (dark preference) a choice index was defined as the time spent in the dark zone of the compartment relative to time spent in the light zone. Consequently, choice index of 1 indicates 100% time in the dark zone, choice index of -1 represents 100% time in the light zone and choice index of 0 represents 50% in the dark and 50% in the light zone.

To measure arousal and visual responsiveness of fish, standard, transparent 6-well plates (34.8 mm in diameter) were used. Illumination and visual stimuli were presented by a screen of a tablet PC from beneath. The choice of time for testing and the applied pharmacological treatment protocols were the same as those in earlier experiments. The experiment consisted of two parts; one was considered as a resting and the other was considered as an acute stress-

affected period. To establish basal conditions, a 20-min habituation phase was introduced before the first part, as zebrafish have been reported to habituate to novelty in this amount of time (Yokogawa et al., 2012). To measure behavior in stress-affected conditions the second part took place immediately after the transfer of animals to a novel compartment. In order to measure arousal state and accompanied sensory responsiveness of the animals, during both parts a black object was projected onto the bottom of the testing compartment that remained stationary for 5 minutes, then started to rotate in circles with its speed increasing 2-fold in every 2 minutes, from 0.5 rpm to 8 rpm, for an overall 10 minutes (see supplementary video 1). Both the initial position and subsequent movement directions of the items were randomized among test subjects. The number of active swimming episodes was measured with a custom-made clustering algorithm based on color clustering (k-means clustering). We considered a swimming episode active if more than 1% pixel change occurred between two consecutive frames. This threshold was calculated empirically from consecutive frames showing stationary or moving animals.

To measure avoidance responses to non-meaningful, e.g. novelty-induced, non-visual challenges, the swimming plus-maze (SPM) test was used, recently developed by our laboratory (Varga et al., 2018). The apparatus is a “+” shaped platform consisting of two plus two opposite arms, different in depth, connected by a center zone. Larval and juvenile zebrafish prefer the deep over the shallow arms and the center zone, a preference linked to anxiety-like motivational states. For the validation procedure and detailed specification of the test see Varga et al. (2018). 10 minutes long behavioral tests were conducted employing the same protocol as described at the LDT. Time spent in each zone and the mean of overall velocity were measured. To characterize arm preference, a choice index was defined as the relative time spent in the deep compared to the time spent in the shallow arms. Consequently, a choice index of 1 indicates 100% time in the deep arms, whereas a choice index of -1

represents 100% time in the more aversive shallow arms and a choice index of 0 represents an equal amount of time in each arm.

To measure avoidance responses to meaningful, e.g. social visual challenges the social preference test of the Dreosti laboratory was used (Dreosti et al., 2015). The test apparatus is a U-shaped platform (40 x 32 mm) consisting of two identical arms, with glass window partitions enabling only visual communication between zebrafish. The test is based on the visually-driven preference of larvae towards conspecifics, a phenomenon emerging around the third week of development. 15-minute-long behavioral tests with the presence of a conspecific were preceded by a 15-minute-long habituation phase according to the protocol from the original paper. Time spent in each zone was measured. To characterize social preference, a choice index was defined as the relative time spent in the arm with the conspecific compared to the time spent in the empty arm. Consequently, a choice index of 1 indicates 100% social preference, a choice index of -1 represents 100% social aversion and a choice index of 0 represents no preference or aversion.

High Performance Liquid Chromatography-tandem mass spectrometry (HPLC/MS/MS)

Zebrafish were terminated and kept at -20 °C until measurements. The body and eyes of each subject were manually removed on ice cold petri dishes, then tissue samples were pooled from the remains of two randomly chosen individuals of the same treatment group. The samples were transferred to Eppendorf tubes and their weight was measured on a semi-micro balance (Precisa EP 225SM-DR). After the addition of 50 µl of 0.1% formic acid solution containing 5% methanol, the samples were homogenized using an ultrasonic sonotrode, and the homogenates were centrifuged to produce protein-free supernatants for HPLC-MS-MS analysis. The separation was carried out by high performance liquid chromatography composed of a Perkin Elmer series 200 high pressure gradient pump, an autosampler, an online degasser and a thermostat. A Phenomenex Synergi Hydro-RP 80 150*3,0 mm, 4 µm

column was used with a gradient elution with methanol (mobile phase A) and 0.1% formic acid (mobile phase B): 0 min to 1.5 min: at 5% A; 3 to 4 min: at 60% A; 4.5 to 7 min: at 5% A. 10 µl samples were injected, and the flow rate was 500 µl/min. Serotonin was detected using a triple quadrupole mass spectrometer (Applied Biosystems MDS SCIEX 4000 Q TRAP) in positive multiple reaction monitoring mode (MRM transition: 177.2 → 170.0).

Immunohistochemistry and confocal microscopy

Zebrafish were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS). For the preparation of cryosections, cryoprotecting dehydration of whole-body samples was carried out in a 10-20-30% sucrose-PBS gradient. Samples were subsequently embedded in Tissue-Tek OCT medium, frozen on dry-ice and stored at -80°C until cryosectioning. 20-µm sections were cut in a Microm HM505 cryostat. Sections were collected onto slides (Thermo Fisher), dried at room-temperature overnight, and stored at -80°C until immunohistochemistry was performed.

For immunohistochemistry, slides with sections were bordered with liquid blocker using a 2mm wide PAP pen (Merck). For each step, 400 µl liquid solution was administered for each slide. Sections were washed three times in PBS (10 min each) and incubated overnight at room temperature with polyclonal anti-5-HT antibody produced in rabbit (1:500, 20080, ImmunoStar) diluted in PBS containing 0.5% bovine serum albumin (A7906, Sigma-Aldrich, Hungary) and 0.25% Triton X-100 (T8787, Sigma-Aldrich, Hungary). Sections were washed three times in PBS and incubated with goat anti-rabbit Alexa Fluor 488 (1:500, 111-545-003, Jackson ImmunoResearch Laboratories Inc.) for 2.5 hours. After several washes in PBS, sections were incubated with DAPI (1:10000, D8417, Sigma-Aldrich, Hungary) for 30 min. Slides were mounted with Mowiol4–88 (81381, Sigma-Aldrich, Hungary) fluorescent mounting medium.

Images were taken using a C2 confocal laser scanning microscope (Nikon Europe, Amsterdam, The Netherlands) using a 20× (Plan Apo VC, NA= 0.75) objective (xy: 0.31μm/pixel). We used 405 (Coherent) and 488 (CVI Melles Griot) lasers, and scanning was done in channel series mode. The pixel dwell time, PMT gain, laser intensity detector, and pinhole settings were kept constant for all image acquisitions.

Image analysis was carried out using the ImageJ software (Schneider et al., 2012). Standard 30 x 30 μm regions of interest (ROI) were chosen and applied in each investigated area. Individual subtract values of a given image were calculated in a neighboring ROI, containing no fibers, by the enhancement of the subtraction to a level in which the background was totally diminished. Subsequently, chosen values were applied to relevant ROIs and the percentage of 5-HT signal in a binary processed picture was measured. Individual data were calculated as means of bilateral measurements.

Experimental design

In *Experiment 1*, we aimed to determine the time point of shift from the light to the dark preferring phenotype during development, representing a specific aspect of the behavioral metamorphosis, by subjecting different sets of zebrafish to the LDT test for 10 minutes at 8, 14 and 28 dpf, respectively. Sampling times were chosen according to the measures of Lau and Guo (2011). To analyze environmental reactivity as well in these developmental stages, we repeated the tests on the subsequent days with the same animals and analyzed habituation capacity (Figure 1a). Different sets of larvae from the same spawning were tested in each 2-day trial set. Between the two tests animals were kept in the isolation tanks described before. Samples sizes were 48 for the 8 dpf, 24 for the 14 dpf and 23 for the 28 dpf age groups.

In *Experiment 2*, we investigated whether chronic absence of social stimuli affects post-metamorphic juvenile behavior and reactivity to acute environmental stimuli (LDT test). We

subjected 14 dpf zebrafish to the LDT test and/or 2 weeks of social isolation, then analyzed their avoidance behavior in the LDT test (Figure 2a) at 29 dpf. Sample sizes were 25 and 21 (naïve and test-experienced) for the socially reared and 15 and 21 (naïve and test-experienced) for the isolated groups.

In *Experiment 3*, we aimed to determine isolation-induced changes in central nervous system 5-HT content under basal and challenge-induced conditions by conducting HPLC-MS measurements on juveniles before, immediately after and 1.5 hours after LDT testing. Sample sizes were 22, 18 and 24 (basal, immediate and 1.5 hours) for the socially reared and 16 for the isolated groups.

In *Experiment 4a*, in order to localize isolation-induced changes in 5-HT responses to acute challenges, we subjected juveniles to the LDT test and analyzed 5-HT immunoreactivity throughout the telencephalon 1.5 hours after the stress exposure. To unravel the connection between the observed behavioral and area-specific physiological changes, we aimed to diminish 5-HT activity by the administration of the anxiolytic agent buspirone, predominantly acting on 5-HT_{1A} autoreceptors (Figure 4a). Sample sizes were 31 and 22 (vehicle and buspirone-treated) for the socially reared and 14 and 16 (vehicle and buspirone treated) for the socially isolated groups.

In *Experiment 4b*, we aimed to investigate the effects of isolation under basal and stress-induced arousal states and accompanying sensory responsiveness by subjecting juveniles to stationary and moving items projected onto the testing arena. To clarify the role of the serotonin response we treated the animals with buspirone between basal and stress-induced phases. Sample sizes were 13 and 14 (vehicle and buspirone-treated) in both socially reared and isolated groups as well.

In *Experiment 4c* and *4d*, we aimed to investigate the effects of isolation in different contexts by subjecting juveniles to the SPM or the U-shaped social preference tests after a single treatment of buspirone. In *Experiment 4c*, sample sizes were 28 and 24 (vehicle and buspirone treated) for the socially reared and 17 and 16 (vehicle and buspirone treated) for the socially isolated groups, while in *Experiment 4d* these were 16 and 25 (vehicle and buspirone-treated) for socially reared and 14 for each isolated group.

Data analysis

Data are shown as mean $\pm$ SEM. We performed statistical analyses in R Statistical Environment (R Core Team, 2017). To analyze the interactional effects of rearing conditions, developmental experiences and pharmacological treatments we used linear mixed models (Julian J. Faraway, 2016) from the *lme4* package (Douglas Bates, 2015). Rearing “social” and treatment “vehicle” were set as reference levels. To separate variance stemming from time or sequence of experimental trials or location of test platforms, these factors were added as random effects to our models. The statistical output of such models indicate if the effects of treatments are identical throughout rearing conditions. In this manner, we only showed the results in relation to the reference category, e.g. the difference between control and treated isolated groups differs from the corresponding difference between socially reared groups. To analyze within-group differences between repeated measures, we fitted linear mixed models with subject identifiers as random effects. We computed Pearson correlation coefficients (*r*) using the *GGally* package (Barret Schloerke et al., 2017). We calculated *p*-values from *t*-values of *lme4* using the *lmerTest* package (Kuznetsova et al., 2016) and rejected H_0 if *p*-values were lower than 0.05.

Results

Behavioral metamorphosis in zebrafish defines a time-window with enhanced environmental reactivity

Since larval and juvenile zebrafish express a different behavioral repertoire (Dreosti et al., 2015; Lau and Guo, 2011) it is evident that, besides the morphological metamorphosis, a behavioral reorganization also occurs in this species. We hypothesized that the time period of such transformation may also function as a window of opportunity for individuals to sample current environmental demands and adapt to potential challenges. Such processes require a great amount of environmental reactivity and may represent a sensitive developmental period that may be suitable to model the effects of early-life experiences. In *Experiment 1* (Figure 1), we aimed to determine the time of the switch from the light to the dark preferring phenotype, representing a specific aspect of the behavioral metamorphosis (Lau and Guo, 2011), by subjecting separate sets of zebrafish to the LDT test for 10 minutes at 8, 14 and 28 dpf respectively (Figure 1a). To characterize environmental reactivity as well in these phases of development, we repeated the experiments the following day (9, 15, or 29 dpf respectively) with the same animals. While 8 dpf larvae preferred the light over the dark zone, the preference was shifted towards the dark zone in older groups, (Figure 1c) outlining the time onset of the aforementioned phenotypic shift. At test repetitions, 9 dpf light-preferring larvae showed increased dark exploration, 15 dpf dark preferring larvae showed increased light exploration, while 29 dpf fish expressed no detectable change of behavior compared to the first testing day (Figure 1d). It is important to note that 15 dpf larvae showed the most extreme inter-test decrease indicated by the slope of change in avoidance (Figure 1d), and this group had the highest proportion of animals showing habituation, 19 out of 24 fish decreased their avoidance (Figure 1e). Furthermore, we did not detect any differences between 14 and 15 dpf test-naïve animals, indicating that the observed effects were experience-dependent

changes and were not biased by aging. Linear regression showed that in the 8 and 14 dpf groups, the absolute value of avoidance in the first tests predicted the absolute value of slope of change in avoidance between tests (Figure 1f). Extreme first-trial avoidance was associated with stronger inter-test changes in behavior. In summary, the first 2 weeks of development mark a specific aspect of behavioral metamorphosis and a period of enhanced reactivity that peaks at the second sampling event. See statistical data in Table 1.

Social isolation in the reactive period disrupts later behavioral responsiveness of zebrafish

Given such increased environmental reactivity around the second week of development, in *Experiment 2* we assessed the effects of different experiences gained in this time-window on the post-metamorphic behavioral phenotype. We subjected zebrafish larvae to acute novelty stress (LDT testing) at 14 and/or chronic environmental isolation from 14 to 28 dpf, then analyzed their avoidance behavior in the LDT test at the juvenile stage (Figure 2a). Note that the chronic environmental deprivation started at the most plastic sampling point where behavioral development has not occurred yet and ended after the morphological metamorphosis, hence we excluded the chance of detecting a possible conservation event of the immature avoidance phenotype. Environmental isolation, regardless of the onset of preliminary LDT testing, decreased avoidance behavior (Figure 2b), the number of transitions between zones (Figure 2d) and mean velocity (data not shown), indicating an overall impairment in responsivity. See statistical data in Table 2.

Social isolation differentially affects central 5-HT levels of zebrafish under resting and novel conditions

The decreased response capacity observed led us to investigate the role of 5-HT in isolation-induced effects, since serotonergic signaling is linked to behavioral constraint and environmental responsiveness as well (for reviews see Brodie and Shore, 1957; Depue and

Spoont, 1986; Lucki, 1998; Soubrié, 1986; Spoont, 1992). To characterize the dynamics of potential 5-HT level changes in our isolation model, we conducted HPLC-MS-MS measurements on whole-brain samples of separate sets of zebrafish under resting conditions, as well as immediately- and 1.5 h after the post-metamorphic LDT test, respectively (Figure 3a). Socially reared fish showed a rapid decrease in 5-HT content in response to LDT testing, changes that were detectable for at least as long as 1.5 hours. In contrast, isolated fish showed decreased baseline levels compared to socially reared conspecifics and a markedly enhanced, transient 5-HT response to LDT testing (Figure 3b). Such differentially directed 5-HT responses to the novelty-challenge were accompanied by a similar decrease in light avoidance that we measured in *Experiment 2*. Interestingly, minute-by-minute analysis revealed that the principal component of such low responsivity was the delayed emergence of avoidance activity, while decreased locomotion was stable throughout the test (Figure 3c and 3d). See statistical data in Table 3.

Social isolation-induced 5-HT changes are specific to the dorsal pallium and, along with behavioral deficits, can be prevented by blockade of 5-HT signaling

In *Experiment 4a*, we assessed the functional connectivity between isolation-induced behavioral and physiological effects. We administered buspirone prior to the post-metamorphic LDT test and used immunohistochemical labeling for 5-HT in several regions of the telencephalon as well (Figure 4c). We hypothesized that such a pharmacological treatment might possibly be able to dampen 5-HT signaling in forebrain areas relevant to the regulation of behavioral processes, since i) buspirone predominantly acts on 5HT1A receptors (Loane, 2012), ii) zebrafish express these mainly as autoreceptors of the raphe nuclei (Norton et al., 2008), which are iii) the only serotonergic cell clusters that project to the teleostean telencephalon (Maximino et al., 2013a), iv) the area that orchestrates dark avoidance behavior (Lau and Guo, 2011). Furthermore, v) buspirone binds to 5HT1A receptors of zebrafish with a

4 times greater affinity than that of rats and an 8 times greater affinity than that of humans (Barba-Escobedo and Gould, 2012). Isolated fish, similarly to those in *Experiment 2* and *3*, showed decreased light avoidance behavior. This effect was preventable by an acute buspirone treatment with a dose that had no effect on socially reared conspecifics (Figure 4b). Immunohistochemical labeling revealed higher 5-HT immunoreactivity in isolated fish compared to controls in the lateral forebrain bundle and in the caudal, but not in any rostral part of the pallium. This difference was abolished by the acute administration of buspirone (Figure 4d and 4e). Taken together, our results display that with a single 5HT1AR agonist treatment we were able to restore the normal novelty-induced avoidance phenotype. In addition, we found 5-HT level differences in fibers that enter the forebrain and target the caudal recess of the telencephalon where cell masses of the dorsomedial and dorsolateral areas, homologue structures of the mammalian amygdala and hippocampus, emerge separately (Wulliman et al., 2012). Such differences were preventable by acute buspirone treatment. In summary, we detected functional connections between forebrain-localized 5-HT response and inadequate avoidance behavior.

Social isolation shifts response capacity between novel and resting conditions and is inverted by the effects of 5-HT signaling blockade

Due to the highly different levels of 5-HT in resting and novel environment, including changes in limbic structures, and ineffective challenge responding of isolated fish, in *Experiment 4b* we assessed possible isolation-induced impairment in stress-induced arousal state. Alertness in mammals is associated with activation of the amygdala (Gläscher and Adolphs, 2003; Harmer et al., 2006) and modulated by 5-HT signaling (Spoont, 1992), hence we speculated that an inadequate emergence of such condition may drive the isolation-induced behavioral impairment of zebrafish. In order to assess this issue, a stationary black slider was projected on the bottom of testing compartments for 5 minutes, which subsequently

started to move in circles with increasing speed (Figure 5a). We sampled behavioral responses in resting conditions, after 20 minutes of habituation, as well as in novel environment, immediately after the transfer to the apparatus. Normally, an animal in novelty-induced alert state react more rapidly to movement or react to a yet slower moving objects (Yokogawa et al., 2012) compared to habituated resting individuals. Socially reared animals, in novel but not in familiar context, showed a decrease in the number of swimming episodes and in the distance from the stationary slider in response to its appearance, while buspirone prevented both of these effects (Figure 5b). Interestingly, isolated fish only showed such stimuli-induced decreased locomotion in response to buspirone treatment. In response to the movement stimulus, socially reared animals increased the frequency of their swimming episodes in novel, but not in resting conditions, while buspirone abolished this reaction (Figure 5c). In contrast, isolated fish showed higher response frequency in resting, and a delayed, oppositely directed response in stress-induced conditions. Buspirone pre-treatment of isolated fish triggered a response similar in shape that of socially reared stressed or isolated habituated animals. In summary, we detected an isolation-induced lack of typical stress-induced, arousal-associated activity in novel, and an exacerbated activity in resting conditions, while buspirone evoked opposite direction effects compared to socially reared animals.

Social isolation-induced decrease in responsiveness is specific to novelty-based visually-driven challenges

Given such isolation-induced low responsivity, potentially driven by an inability to form stress-associated arousal, next we investigated whether the effects are challenge-specific. In order to assess this issue, in Experiment 4c and 4d, we analyzed isolation-induced changes in response to different environmental challenges, such as the swimming plus-maze (Varga et al., 2018) and the U-shaped social preference tests (Dreosti et al., 2015). Similarly to the LDT test, both tests trigger approach-avoidance conflict in the animal, however, presenting

different type of stimuli, e.g. non-visual non-meaningful (novelty) and visual meaningful (social) challenges. To analyze the potential role of 5-HT signaling as well, we administered buspirone prior to the tests (Figure 6a). Interestingly, isolated fish showed increased avoidance compared to socially reared controls in both tests, however, buspirone completely (Figure 6b) or partially (Figure 6c) prevented such effects. Note, that in the case of the social preference test, buspirone abolished social activity regardless of its direction, e.g. the preference in case of socially reared and the aversion in case of isolated animals. These results indicate that decreased responsiveness of isolated animals is specific to visual non-meaningful, novel stimuli, e.g. the light/dark or the sensory-motor reaction tests.

Discussion

We have characterized a period during zebrafish development that is highly reactive to the environment in terms of the ability of the individual to change behavioral responses to a repeated challenge. Absence of social stimuli during this period promoted an atypical challenge-coping phenotype, characterized by exacerbated alertness under resting conditions and inability to show novelty-induced arousal and avoidance behavior. In line with these behavioral impairments, isolated fish expressed lower baseline, and same magnitude, but oppositely directed serotonin-level changes in response to novelty compared to socially reared subjects. This effect was localized in the caudal excess of the dorsal pallium, a brain area responsible for adequate stress-induced aversion. 5HT1AR agonist treatment prevented isolation-induced serotonin response in the forebrain and rescued physiological challenge-induced behaviors as well, indicating a functional connection between these processes. The effects were specific to visual challenges in a novel context, since they were undetectable at non-visual novelty, and visual social challenges.

In our first experiments, we showed that a switch from dark to light avoidance, a specific aspect of the behavioral metamorphosis, occurs between the first and second week of

development in zebrafish. Furthermore, we showed that this process overlaps with the ability to decrease avoidance in response to repeated challenge, suggesting that this switch is accompanied by enhanced behavioral plasticity, thus it may serve as a tool to model early life experience-dependent phenomena. The light/dark choice reversal was first observed by Lau and Guo (2011), who estimated its occurrence later than we did, between the second and third week of development. This discrepancy indicates a potential experience-dependent flexibility in the onset of such process, while such variability in the onset supports our hypothesis that the first month of ontogenesis possibly represents a highly plastic, reactive developmental phase. Decreased avoidance at repeated testing supports this idea as well, as it may be driven by habituation, a neuroplasticity-based process (Thompson and Spencer, 1966). The idea of habituation is supported by our findings, as i) decreased avoidance was detectable regardless of the direction of baseline preference, ii) it was not caused by aging, and iii) stronger baseline preference predicted a stronger change in its expression. In summary, one aspect of the behavioral metamorphosis coincides with and speculatively requires a peak in adaptive capacity.

In our next experiment, we showed that an acute challenge, in contrast to its short-term effect, does not influence avoidance 2 weeks later, indicating a spontaneous recovery from novelty stress during the long inter-test period. In contrast, absence of social stimuli during this sensitive period exerted robust effects on behavior, including decreased avoidance and locomotion. Since the LDT test, used here to mimic an acute environmental challenge, is a pharmacologically (Chen et al., 2015; Gould, 2011; Maximino, 2011), physiologically (Chen et al., 2015) and behaviorally (Blaser, 2011) validated tool for measuring anxiety-associated behavior of zebrafish, such decreased avoidance might be interpreted as social isolation-induced anxiolysis. This theory, however, is unlikely, as i.) application of the anxiolytic agent buspirone prevented the decrease, thus, if the ‘anxiolysis hypothesis’ is accepted, it acted as

an anxiogenic drug, contradicting its widely accepted effect on behavior; ii.)

phenomenological correlates of other anxiety-like domains were enhanced in response to social isolation. According to rodent and zebrafish literature, it is rare, but not unique (Parker, 2012; Shams et al., 2015), that such a chronic environmental perturbation as social isolation promotes a low anxiety phenotype (for reviews see Fone and Porkess, 2008; Hall, 1998; Lukkes et al., 2009), particularly, several authors discussed social isolation of zebrafish as beneficial, since it consistently decreased stress-induced cortisol response and in some cases avoidance as well (Forsatkar et al., 2017; Giacomini, 2015; Parker, 2012; Shams et al., 2015). Despite the importance of these results, we suggest a more careful interpretation of the above data. Clinical and preclinical data suggest that an inadequate cortisol response may drive or at least coincide with abnormal behavior, regardless of the direction in which it moves out of the physiological range (Gutiérrez et al., 2019; Haller et al., 2001; McBurnett et al., 2000; Toth et al., 2011; van Goozen et al., 1998; Vanyukov et al., 1993). Furthermore, the corticosteroid response is not necessarily in a positive or in any relation with the expression of anxiety-like behavior (Aliczki et al., 2013; Mikics et al., 2005). On the other hand, isolation-induced low avoidance has also been interpreted as reduced pro-active coping and described as a stable “slow-emerging” phenotype in rodents (Arakawa, 2007, 2005). Isolated rats consistently showed a higher latency to enter the centrum of an unfamiliar open field arena, or a lower frequency of entries into the centrum of a familiar but brightly lit open field arena, which indicates that isolation-induced delayed exploration is related to adversity and the accompanying stress-response. In another study, socially reared zebrafish individuals showing delayed emergence from a dark to a brightly lit arena, a phenotype resembling the one observed in our study, showed lower locomotion in general and lower challenge-induced but similar baseline cortisol responses (Tudorache et al., 2015). One can hypothesize that a low stress-induced cortisol response, without any change in basal levels, and the accompanying

low avoidance behavior might indicate a malfunction of challenge-responding. This phenotypic background may account for low avoidance in adult and decreased locomotion in 30 dpf juvenile zebrafish after social isolation as well, observed by Shams and colleagues (Shams et al., 2017a, 2015). This theory is also supported by our findings on isolation-induced low, delayed avoidance and inadequate arousal in response to a challenge and enhanced arousal under baseline, resting conditions. This indicates that isolated fish shift stress-associated alertness to baseline conditions and in this manner are unable to develop adequate internal states and accompanying behavioral responsiveness in neither of the contexts. This also means that lack of stimuli during the characterized, highly plastic developmental period has a crucial role in shaping the post-metamorphic behavioral phenotype.

Given the low responding behavioral phenotype, we assessed isolation-induced effects on 5-HT signaling since it is highly sensitive to early life adversity and is known as a prominent modulator of mood, as well as general or specific arousal states (Lucki, 1998; Soubrié, 1986; Spont, 1992). We found that isolated fish show decreased baseline 5-HT tone and, contrarily to socially reared conspecifics, a rapid gradual 5-HT increase in response to the LDT challenge. This difference was non-significant 1.5 hours later on a whole-brain level, however, it was still detectable in fibers projecting to pallial areas, e.g. the lateral forebrain bundle and the caudal recess of the dorsal pallium. Furthermore, 5HT1AR agonist treatment prevented differences in forebrain 5-HT immunoreactivity and behavior as well. Our findings regarding brain 5-HT levels are in contradiction with previous reports, however, this discrepancy might be explained by the facts that in these papers either i.) only extracellular 5-HT content was measured and ii) adult animals were employed (Maximino et al., 2013b) or iii) social isolation was conducted in adult fish or in larvae from the age of 0 dpf (Shams et al., 2017a, 2017b, 2015). The latter case, together with rodent data supports that the period

during which isolation occurs is fundamental regarding later consequences, highlighting the importance of critical and sensitive developmental periods (Einon and Morgan, 1977; Fone and Porkess, 2008; Hall, 1998; Lukkes et al., 2009a). We suggest that the environmental perturbation we applied in a period characterized by enhanced adaptive capacity exerts neurochemical and behavioral effects that overtop the ones previously observed by other authors. It is also important to note that besides the discrepancy between our results and data from other laboratories employing zebrafish models, numerous clinical and preclinical rodent studies support our findings: several authors described 5-HT tone as a general inhibitor of responsiveness, which decreases in response to sudden environmental changes allowing the expression of adequate behavioral responses. From this perspective, 5-HT acts as a context-dependent modulator that levels responsiveness into non-impulsive ranges (Brodie and Shore, 1957; Depue and Spoont, 1986; Lucki, 1998; Soubrié, 1986; Spoont, 1992). Behrendt suggests that 5-HT downregulates responsivity partially through its actions on amygdala function (Ralf-Peter Behrendt, 2011), which theory is supported by several human fMRI studies investigating the mechanisms of selective 5-HT reuptake inhibitors in aversive contexts (Del-Ben et al., 2005; Harmer et al., 2006; Murphy et al., 2009). In addition, isolation-induced enhanced serotonergic activity and fiber density in the amygdala were also reported in most rodent neurochemistry studies (Gos et al., 2006; Günther et al., 2008; Lehmann et al., 2003), in line with our current results.

In summary, we suggest that 5-HT tone of zebrafish restricts behavior under resting conditions, while its phasic decrease yields behavioral stress responses during a challenge. Social isolation during an adaptive time-window leads to an abnormal 5-HT phenotype in juveniles, with serotonin levels inversely reflecting those of socially reared ones, thus exerting shifted responsivity as well. The phenomenon is modulated in the caudal recess of the telencephalon, where homologue structures of the mammalian amygdala and hippocampus are

located. Our findings indicate that zebrafish possess a conserved serotonergic mechanism that context-dependently modulates environmental reactivity and is highly sensitive to early life experiences.

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Figure legends

Figure 1: a) Protocol of *Experiment 1*. Red timeline shows the age of larvae expressed in days post fertilisation (dpf). Individual black lines show different groups of larvae. **b)** Schematic figure of the LDT apparatus. **c)** Avoidance behavior of zebrafish at the 1st testing day throughout the first month of development. Left axis shows the percentage of time, right axis shows choice index in which 1 indicates 100% time in the dark zone, whereas -1 represents 100% time in the light zone. Asterisk indicates significant difference compared to 8 dpf age group. **d)** Slope of change in avoidance behavior between the two testing days. Asterisk indicates significant difference compared to 8 dpf age group. **e)** Individual values of choice indices in the 14-15 dpf age group and test naïve age control. Dashed lines mark increased avoidance, solid lines indicate decreased avoidance. Asterisk indicates significant difference from 1st testing day. **f)** Relation between the first and second days of testing. Trends are indicated by solid regression lines and 95% confidence intervals. Black asterisk indicates significant linear regression. Orange asterisk indicates marginal significance ($p=0.0583$).

Figure 2: a) Protocol of *Experiment 2* (top) and schematic drawing of the isolation tanks (bottom). Red timeline shows the age of larvae expressed in days post fertilisation (dpf). **b)** Avoidance behavior of post-metamorphic zebrafish 2 weeks following a single LDT challenge and/or after 2 weeks of chronic social isolation. Left axis shows the percentage of time, right axis shows choice index in which 1 indicates 100% time in the dark zone, whereas

–1 represents 100% time in the light zone. The reference levels of the linear mixed model were „social rearing” and „treatment none”. Asterisk indicates differences between rearing conditions at the level „treatment none”, n.s. indicates no significant differences between the relation of the treatment levels in socially reared and isolated groups. **c)** Number of transitions between light and dark zones of post-metamorphic zebrafish 2 weeks following a single LDT challenge and/or after 2 weeks of chronic social isolation.

Figure 3: a) Protocol of *Experiment 3*. Red timeline shows the age of larvae expressed in days post fertilisation (dpf). **b)** Whole-brain 5-HT concentration of post-metamorphic zebrafish in resting conditions, and immediately and 1.5 hours after LDT challenge. Asterisk indicates differences between groups at the level of a single condition (basal level, LDT, LDT+1.5 h), hashtag indicates significant group*condition interaction. **c)** Minute-by-minute resolution analysis of avoidance measured by choice index and **d)** velocity. Hashtag indicates significant group*time interaction.

Figure 4: a) Protocol of *Experiment 4a*. **b)** Avoidance behaviour of post-metamorphic zebrafish 2 weeks following chronic social isolation and/or after acute buspirone treatment. Left axis shows the percentage of time, right axis shows choice index in which 1 indicates 100% time in the dark zone, whereas –1 represents 100% time in the light zone. Reference levels of the linear mixed model were „social rearing” and „treatment vehicle”. Asterisk indicates differences between rearing conditions at the level of treatment vehicle, hashtag indicates significant differences between the relation of the treatment levels in socially reared and isolated groups. **c)** Schematic drawing (top) and confocal microphotographs (maximum intensity projection) (bottom) of the investigated areas. The image was taken from a whole-brain preparatum of a zebrafish. Dashed lines indicate the approximate antero-posterior position of the investigated areas. **d)** Quantification of 5-HT immunoreactivity in the analyzed brain regions. Asterisk indicates differences between rearing conditions at the level of

treatment vehicle, hashtag indicates significant differences between the relation of the treatment levels in socially reared and isolated groups. **e)** Representative confocal microphotographs (maximum intensity projection) of sites with significant differences in immunoreactivity.

Figure 5: a) Protocol of *Experiment 4b*. Red timeline shows the age of larvae expressed in days post fertilisation (dpf). **b)** Locomotor response to (top) and the total distance from (bottom) the projected stationary object in resting and novel conditions and in response to prior buspirone treatment. Reference levels of the linear mixed model were „social rearing” and „condition resting”. Asterisk indicates differences between experimental conditions (resting, novelty, novelty + buspirone) at the level of each rearing conditions. Dashed lines indicate $\pm$ standard error of mean of activity during the habituation period. **c)** Minute-by-minute resolution analysis of locomotor response to the moving object in resting and novel conditions and in response to prior buspirone treatment. The number of active swimming episodes are presented relative to previous (prestim) activity of the individuals, band shows $\pm$ standard error of mean. Grey gradient scale represents the dynamics of speed increase of the moving object. Dashed lines mark standard errors of means of the prestimulus activity. Asterisk represents significant differences from resting condition. **d)** Arousal profiles of socially reared and isolated animals presented as locomotor responses to the moving object under different conditions (resting, novelty, novelty + buspirone). 3 dimension starplot shows the 3 different conditions. Locomotor response is presented as the area under the curve of active episodes relative to the previous activity of the individuals.

Figure 6: a) Protocol of *Experiment 4c* and *4d*. Red timeline shows the age of larvae expressed in days post fertilisation (dpf). **b)** Surface avoidance behavior of post-metamorphic zebrafish 2 weeks following chronic social isolation and/or acute buspirone treatment (top) and their representative heatmaps (bottom). Left axis shows the percentage of time, right axis

shows choice index in which 1 indicates 100% time in the deep zone, whereas -1 represents 100% time in the shallow zone. The reference levels of the linear mixed model were „social rearing” and „treatment vehicle”. **c)** Social preference of post-metamorphic zebrafish 2 weeks after chronic social isolation and/or after buspirone treatment (top) and their representative heatmaps (bottom). Left axis shows the percentage of time, right axis shows choice index in which 1 indicates 100% time in the social chamber, whereas -1 represents 100% time in the empty chamber. The reference levels of the linear mixed model were „social rearing” and „treatment vehicle”. Asterisk indicates significant differences from socially reared vehicle treated animals, hashtag indicates significant differences between the relation of the treatment levels in socially reared and isolated groups.

Table legends

Table 1: Statistical data of *Experiment 1*. ^{\$} The statistical output of these linear mixed models are able to show significant differences from a reference condition in each analyzed levels, i.e. age and testing day. Age “8 dpf” and testing day “1st” were set as reference conditions. We also show interactional effects in relation to the reference categories, e.g. the difference between the 1st and 2nd tests (~) at 8 dpf differ from the corresponding difference between the 14 dpf age groups. Note that different hypotheses testing methods were used, hence shown parameters may differ from method to method.

Table 2: Statistical data of *Experiment 2*. ^{\$} The statistical output of these linear mixed models are able to show significant differences from a reference condition in each analyzed levels, i.e. rearing condition and prior testing. Rearing “social” and prior testing “no LDT” were set as reference conditions. We also show interactional effects in relation to the reference categories, e.g. the difference between the “no LDT” and “LDT” groups (~) in socially reared groups do not differ from the corresponding difference between the isolated groups.

Table 3: Statistical data of *Experiment 3*. Note that different hypotheses testing methods were used, hence shown parameters may differ from method to method.

Table 4: Statistical data of *Experiment 4a*. ^{\$} The statistical output of these linear mixed models are able to show significant differences from a reference condition in each analyzed levels, i.e. rearing and treatment. Rearing “social” and treatment “vehicle” were set as reference conditions. We also show interactional effects in relation to the reference categories, e.g. the difference between the treatment vehicle and buspirone (~) in socially reared groups differ from the corresponding difference between the isolated groups. Note that different hypotheses testing methods were used, hence shown parameters may differ from method to method.

Table 5: Statistical data of *Experiment 4b, 4c and 4d*. ^{\$} The statistical output of these linear mixed models are able to show significant differences from a reference condition in each analyzed levels, i.e. rearing and treatment/condition. Rearing “social” and treatment/condition “resting” were set as reference conditions. We also show interactional effects in relation to the reference categories, e.g. the difference between the resting and novelty condition (~) in socially reared groups do not differ from the corresponding difference between the isolated groups. Note that different hypotheses testing methods were used, hence shown parameters may differ from method to method.

a

b

c

d

e

Table 1

Experiment	Measure	Test statistics	Reference condition ^s	Compared group	Estimate	SEM	n	t-value	p-value
1	choice index	linear mixed model	8 dpf/1 st test	14 dpf/1 st test	0.892	0.119	48 vs 24	7.516	2.48e-12
			8 dpf/1 st test	27 dpf/1 st test	0.756	0.120	48 vs 23	6.285	2.35e-09
			8 dpf/1 st test	8 dpf/2 nd test	0.256	0.098	48	2.630	0.00927
			8 dpf/1 st test ~	14 dpf/1 st test ~	-0.769	0.168	-	-4.587	8.36e-06
			8 dpf/2 nd test	14 dpf/2 nd test					
			8 dpf/1 st test ~	27 dpf/1 st test ~	-0.155	0.170	-	-0.912	0.36306
	-	linear regression	8 dpf slope (abs value)	8 dpf choice index	0.426	0.111	48	3.825	0.00042
			14 dpf slope (abs value)	14 dpf choice index	0.524	0.262	24	1.997	0.0583
			27 dpf slope (abs value)	27 dpf choice index	0.195	0.286	23	0.681	0.50332
	Measure	Test statistics	Factor		Sum sq	Mean sq	n	F-value	p-value
	slope	ANOVA	age group		9.45	4.723	-	12.87	1.24e-05
	Measure	Test statistics	Compared groups		Estimate	-	n	-	p-value
	slope	Tukey post-hoc	8 dpf	14 dpf	-0.743		48 vs 24		0.00002
			8 dpf	27 dpf	-0.043	-	48 vs 23	-	0.95924
			14 dpf	27 dpf	0.700		24 vs 23		0.00044

Table 2

Experiment	Test statistics	Reference condition ^s	Compared group	Measure	Estimate	SEM	n	t-value	p-value
2	Linear mixed model	soc/no LDT	soc/LDT	choice index	-0.208	0.207	25	-1.005	0.31826
				zone transitions	-0.286	3.775	21	-0.076	0.93973
		soc/no LDT	iso/LDT	choice index	-0.530	0.186	25	-2.853	0.00564
				zone transitions	-9.772	3.615	15	-2.704	0.00842
		soc/no LDT ~	iso/no LDT ~	choice index	0.338	0.283	-	1.193	0.23664
		soc/LDT	iso/LDT	zone transitions	1.4117	5.3611	-	0.263	0.79300

Table 3

Experiment	Measure	Test statistics	Factor	Sum sq	Mean sq	n	F-value	p-value		
3	5-HT cc	two-way ANOVA	condition	22603	11301	-	12.624	3.65e-05		
			group	705	705		0.788	0.379		
			condition*group	24448	12224		13.655	1.86e-05		
		Test statistics	Compared groups	Estimate	-	n	-	p-value		
		Tukey post-hoc	soc/basal	soc/LDT	-56.810		22 vs 18		0.00135	
			soc/basal	soc/LDT+1.5h	-75.748		22 vs 24		0.00001	
			soc/LDT	soc/LDT+1.5h	-18.938		18 vs 24		0.70561	
			iso/basal	iso/LDT	45.029		16 vs 16		0.04450	
			iso/basal	iso/LDT+1.5h	-5.132	-	16 vs 16	-	0.99933	
			iso/LDT	iso/LDT+1.5h	-50.161		16 vs 16		0.01807	
			soc/basal	iso/basal	50.512		22 vs 16		0.00820	
			soc/LDT	iso/LDT	-51.327		18 vs 16		0.01100	
			soc/LDT+1.5h	iso/LDT+1.5h	-20.104		24 vs 16		0.68317	
	Measure	Test statistics	Reference condition*	Compared group	Estimate	SEM	n	t-value	p-value	
	choice index	repeated measures ANOVA	iso/timebin1	soc/timebin1	0.309	0.161	34 vs 46	1.916	0.05763	
			iso/timebin1	iso/timebin subsequent	0.027	0.010		2.626	0.00882	
			iso/timebin1	soc/timebin1	-0.029	0.013		-2.181	0.02948	
			~ iso/timebin	soc/timebin subsequent						

Table 4

Experiment	Measure	Test statistics	Reference condition ^s	Compared group	Estimate	SEM	n	t-value	p-value
4a	choice index	Linear mixed model	soc/veh	soc/busp	0.0210	0.142	31 vs 22	0.148	0.8826
			soc/veh	iso/veh	-0.335	0.155	21 vs 14	-2.159	0.0340
			~	~	0.664	0.224	-	2.963	0.0041*
			soc/busp	iso/busp					
	Measure	Test statistics	Factor		Sum sq	Mean sq	n	F-value	p-value
	5HT (Pc)	two-way ANOVA	treatment		20.94	20.94	-	3.758	0.06171
			group		43.75	43.75		7.851	0.00868
			treatment*group		3.68	3.68		0.661	0.42250
		Test statistics	Compared groups		Estimate	-	n	-	p-value
		Tukey post-hoc	soc/veh	soc/busp	0.952	-	9 vs 6	-	0.88177
			soc/veh	iso/veh	-2.758		9 vs 12		0.04839
			soc/veh	iso/busp	-0.438		9 vs 7		0.97931
			soc/busp	iso/veh	-3.710		6 vs 12		0.02888
			soc/busp	iso/busp	-1.390		6 vs 7		0.73174
			iso/veh	iso/busp	-0.438		12 vs 7		0.97931
	Measure	Test statistics	Factor		Sum sq	Mean sq	n	F-value	p-value
	5HT (lfb)	two-way ANOVA	treatment		16.54	16.54	-	2.287	0.1400
			group		22.27	22.27		3.079	0.0886
			treatment*group		32.21	32.21		4.452	0.0425
		Test statistics	Compared groups		Estimate	-	n	-	p-value
		Tukey post-hoc	soc/veh	soc/busp	-1.116	-	8 vs 6	-	0.89567
			soc/veh	iso/veh	-3.001		8 vs 12		0.05622
			soc/veh	iso/busp	0.002		8 vs 10		1.00000
			soc/busp	iso/veh	-1.884		6 vs 12		0.61549
			soc/busp	iso/busp	1.118		6 vs 10		0.89518
			iso/veh	iso/busp	3.003		12 vs 10		0.05600
	Measure	Test statistics	Factor		Sum sq	Mean sq	n	F-value	p-value
	5HT (Pr)	two-way ANOVA	treatment		1.789	1.789	-	1.273	0.27192
			group		12.175	12.175		8.664	0.00776
			treatment*group		3.321	3.321		2.363	0.13917
		Test statistics	Compared groups		Estimate	-	n	-	p-value
		Tukey post-hoc	soc/veh	soc/busp	-1.269	-	8 vs 6	-	0.36951
			soc/veh	iso/veh	0.611		8 vs 7		0.83414
			soc/veh	iso/busp	0.850		8 vs 6		0.66761
			soc/busp	iso/veh	1.880		6 vs 7		0.03660
			soc/busp	iso/busp	2.119		6 vs 6		0.02015
			iso/veh	iso/busp	0.239		7 vs 6		0.97946
	Measure	Test statistics	Factor		Sum sq	Mean sq	n	F-value	p-value
	5HT (Sr)	two-way ANOVA	treatment		0.150	0.150	-	0.289	0.596
			group		0.491	0.490		0.942	0.342
			treatment*group		0.012	0.012		0.024	0.878

Table 5

Experiment	Stimulus	Measure	Test statistics	Reference condition [§]	Compared group	Estimate	SEM	n	t-value	p-value		
4b	stationary	swimming episodes	linear mixed model	soc/resting	iso/resting	-8.141	10.605	27 vs 27	-0.768	0.4447		
		distance from item				-5.355	25.217		-0.212	0.8323		
	moving	swimming episodes				-5.326	6.786		-0.785	0.4328		
	stationary	swimming episodes		soc/resting	soc/busp	-4.074	12.377	27 vs 14	-0.329	0.7430		
		distance from item				-10.613	28.864		-0.368	0.7143		
	moving	swimming episodes				2.498	8.531		0.293	0.7698		
	stationary	swimming episodes		soc/resting	soc/stressed	-44.972	11.602	27 vs 13	-3.876	0.0002		
		distance from item				-55.982	27.018		-2.072	0.0424		
	moving	swimming episodes				17.476	8.341		2.095	0.0366		
	stationary	swimming episodes		soc/resting ~ soc/busp	iso/resting ~ iso/busp	-29.634	16.617	-	-1.783	0.0791		
		distance from item				13.560	38.691		0.350	0.7272		
	moving	swimming episodes				-7.286	11.587		-0.629	0.5298		
stationary	swimming episodes	soc/resting ~ soc/stressed	iso/resting ~ iso/stressed	32.381	16.804	-	1.927	0.0582				
	distance from item			63.515	39.125		1.623	0.1095				
moving	swimming episodes			3.931	12.362		0.318	0.7506				
Experiment	Test	Measure	Test statistics	Reference condition*	Compared group	Estimate	SEM	n	t-value	p-value		
4c	SPM	choice index	Linear mixed model	soc/veh	iso/veh	-0.237	0.120	28 vs 17	-1.984	0.0506		
				soc/veh	soc/busp	0.026	0.108	28 vs 24	0.240	0.8111		
				soc/veh ~ soc/busp	iso/veh ~ iso/busp	0.287	0.173	-	1.661	0.1007		
4d	sociability			soc/veh	iso/veh	-0.790	0.208	16 vs 17	-3.802	0.0003		
				soc/veh	soc/busp	-0.435	0.182	16 vs 25	-2.396	0.0194		
				soc/veh ~ soc/busp	iso/veh ~ iso/busp	0.703	0.292	-	2.404	0.0191		