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ORIGINAL ARTICLE

Paternal exposure to excessive methionine altered behavior and neurochemical activities in zebrafish offspring

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Abstract

An increase in plasma L-methionine (Met) levels, even if transitory, can cause important toxicological alterations in the affected individuals. Met is essential in the regulation of epigenetic mechanisms and its influence on the subsequent generation has been investigated. However, few studies have explored the influence of a temporary increase in Met levels in parents on their offspring. This study evaluated the behavioral and neurochemical effects of parental exposure to high Met concentration (3 mM) in zebrafish offspring. Adult zebrafish were exposed to Met for 7 days, maintained for additional 7 days in tanks that contained only water, and then used for breeding. The offspring obtained from these fish (F1) were tested in this study. During the early stages of offspring development, morphology, heart rate, survival, locomotion, and anxiety-like behavior were assessed. When these animals reached the adult stage, locomotion, anxiety, aggression, social interaction, memory, oxidative stress, and levels of amino acids and neurotransmitters were analyzed. F1 larvae Met group presented an increase in the distance and mean speed when compared to the control group. F1 adult Met group showed decreased anxiety-like behavior and locomotion. An increase in reactive oxygen species was also observed in the F1 adult Met group whereas lipid peroxidation and antioxidant enzymes did not change when compared to the control group. Dopamine, serotonin, glutamate, and glutathione levels were increased in the F1 adult Met group. Taken together, our data show that even a transient increase in Met in parents can cause behavioral and neurochemical changes in the offspring, promoting transgenerational effects.

Keywords Behavior · Hypermethioninaemia · Methionine · Transgenerational · Zebrafish offspring

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Introduction

Homeostasis in the composition of intracellular and extracellular amino acids requires an efficient mechanism of cell regulation that prevents diseases (Bröer and Bröer 2017). Imbalances in plasma amino acid levels promote neurological changes that affect mood, anxiety, aggression, and social behavior (Le Floc'h et al. 2011; Hrncic et al. 2016; Gulsun et al. 2016; de Lima et al. 2017). L-Methionine (Met) is an amino acid that acts directly or indirectly, through methylation processes, in the formation of several other molecules, including amino acids, such as glutathione, carnitine, and taurine, and neurotransmitters, including dopamine, epinephrine, and serotonin (Martínez et al. 2017). However, conditions that elevate Met plasma levels to toxic concentrations characterize a pathology known as hypermethioninaemia. This condition can occur through non-genetic causes, including a high protein diet, hepatic disease or premature birth, or genetic conditions that are related to an inborn

error amino acid metabolism, with impairment of different enzymes (Mudd 2011). Although it may be asymptomatic, hypermethioninaemia can cause myopathy, hypotonia, and neurological problems (dystonia, tremor, and cognitive deficits) (Schweinberger and Wyse 2016). Studies have shown that even a transient increase in Met levels is responsible for causing toxicological changes, such as extremity tremor, microcephaly, developmental delay, anxiety, and liver disorders (Tsuchiyama et al. 1982; Chang et al. 2019).

The homeostasis in the amino acid cycle is considered even more primordial during pregnancy. The rapid growth and development of the fetus during pregnancy requires balanced amounts of protein (Elango and Ball 2016; Manta-Vogli et al. 2020). Amino acids play essential roles in protein synthesis and activation of cellular functions that are required for a successful pregnancy (Manta-Vogli et al. 2020). Thus, unbalanced amino acid metabolism can generate serious consequences for the fetus, low birth weight, and increase neonatal mortality (Prick et al. 2011; Blumfield and Collins 2014). Within this context, epigenetic changes have been discussed as one of the possible mechanisms for several neurological diseases of subsequent generations caused by an unbalanced nutritional profile (Burdge and Lillycrop 2010). Within the 20 main amino acids, Met plays a crucial role in epigenetic maintenance: its derivative S-adenosylmethionine (SAM-e) can donate its methyl group to a variety of molecules, including DNA and histones (Tang et al. 2017; Zhang 2018). However, the impact of disturbed Met metabolism during pregnancy is still poorly understood (Rees et al. 2006) and the effect on the zebrafish offspring of a transient increase in Met levels in parents is unknown.

Previous studies in a zebrafish model of hypermethioninaemia (3 mM Met) showed increased acetylcholinesterase activity and accumulation of glutamate and adenosine triphosphate (ATP), a change that may exert a potentially damaging effect on the nervous system and thus cause neurotoxicological consequences (e.g., cognitive impairment) (Vuaden et al. 2012, 2016). Besides, high Met levels induce oxidative stress and inhibit the Na^+, K^+-ATPase activity in rat hippocampus; such effects may be related to the neurophysiopathology observed in patients with severe hypermethioninaemia (Stefanello et al. 2007b). Currently, with a focus on assessing the influence of amino acid imbalances on subsequent generations, some studies are exploring the effects of high Met levels on behavior and cognition in the offspring, as well as the mechanisms involved in these alterations (Ryan et al. 2018; Schweinberger et al. 2018). Methyl donor supplemented (Met and cysteine) fathers displayed transgenerational effects on learning and behavior in offspring mice (Ryan et al. 2018). The subcutaneous administration of Met during gestation in rats has important effects on the offspring, such as decreased Na^+, K^+-ATPase and Mg^{2+}-ATPase activities, changes in the brain oxidative state, and consequent impairment of short and long-term memories. Therefore, maternal hypermethioninaemia may be a predisposing factor for brain damage during intrauterine life (Schweinberger et al. 2014, 2017).

Several studies have investigated innate errors in the metabolism of amino acids in zebrafish (Vuaden et al. 2012; Capiotti et al. 2013; Savio et al. 2013; Wager et al. 2014; Quintana et al. 2017). This species is an increasingly utilized animal model for translational studies due to the high genetic homology and conservation of its metabolism with humans (Barbazuk et al. 2000). In addition, zebrafish have similarities in epigenetic regulation with a relatively short generational period and sexual maturity occurring around 3–4 months (Horzmann and Freeman 2018). Therefore, this animal model is suitable to address the transgenerational effects of high Met concentration exposure. Given that the mechanisms involved in these effects are unclear, this study evaluated the influence of parental exposure to high Met concentration on the morphology, locomotion, anxiety, social interaction, aggression, and memory of the zebrafish offspring during the larval and adult stages. We also analyzed the oxidative status, amino acids, and neurotransmitter levels to better understand the mechanisms involved in the transgenerational effects induced by excessive Met exposure.

Materials and methods

For this study, a total of 888 wild-type zebrafish from the AB background were used in the larval stage [4–12 days post-fertilization (dpf)] and in the adult stage (5–6 months, 0.2–0.4 g). The parents were taken from our breeding colony. Until the treatment, the parents were maintained in recirculating systems (Zebtec, Tecniplast, Italy) with equilibrated filtered water. The water temperature was maintained at 28 ± 2 °C, pH at 7.0 ± 0.5, conductivity at 300–700 µS, total ammonia at < 0.02 mg/L, nitrite at < 1 mg/L, nitrate at < 50 mg/L, and chloride at 0 mg/L. Animals were subjected to a natural (14 h light:10 h dark) photoperiod. The animals were feed with paramecium between 6 and 14 dpf; and after 14 dpf, they received commercial flakes fish food (TetraMin Tropical Flake Fish®) supplemented with brine shrimp three times a day (Westerfield 2007). The offspring in the adult stage were maintained in tanks with water in the same conditions. The animals were submitted to the analysis 1 h after the usual feeding, except for the inhibitory avoidance test in which the animals were not feeding.

To obtain the offspring (F1), we used breading tanks (Tecniplast, Italy). During the night, the parents were separated in this tank in a proportion of 1:2 female-to-male ratio (Westerfield 2007). In the morning when the laboratory lights were turned on, the transparent barrier was removed, and the animals remained for 1 h together. The embryos

were collected and placed in Petry dishes, 1 larva per 7 mL and kept in a Biochemical Oxygen Demand (BOD) incubator up to 14 dpf. After 14 dpf, they were transferred to a tank with a density of 1 larva per 60 mL. From 30 dpf until adulthood, the animals were maintained at a density of 1 animal per 200 mL. This study was approved by the Comissão de Ética no Uso de Animais of Pontifícia Universidade Católica do Rio Grande do Sul (3758- CEUA- PUCRS) and was registered in the Sistema Nacional de Gestão do Patrimônio Genético e Conhecimento Tradicional Associado—SISGEN (Protocol No. A3B073D). The sample size of our experiments was calculated based on previous studies with zebrafish (Altenhofen et al. 2017a; Nabinger et al. 2018; Mocelin et al. 2019; Bevilaqua et al. 2020).

Treatments

The animal parents were chosen randomly and separated into control and Met-treated groups. The animals were moved to a glass tank with 5 L, considering 3 animals per 1 L and were exposed to 3 mM of L-Met (L-Met, C5H11NO2S, M9625, Sigma-Aldrich Ltda, Brazil) or water (control group) for 7 days. To maintain the Met concentration, the aquarium water was exchanged on days 3 and 5 (Vuaden et al. 2012). The dose was chosen according to studies about hypermethioninaemia in rodents (Stefanello et al. 2007a) and zebrafish (Vuaden et al. 2012, 2016). For the analyses of Met concentrations in water, we collected samples before and after water preparation and evaluated the Met by liquid chromatography coupled to mass spectrometry (LC–MS/MS). After 7 days, the parents were placed in breeding tanks to obtain the offspring. The parent's Met exposure was performed in six different tanks and from each exposure tank were performed 5 matings (2 males:1 female). However, the number of eggs was insufficient for subsequent experiments. Thus, we maintained the parents for 7 days in tanks that contained only water. Subsequently, the animals were bred, and the eggs were collected. The embryo offspring (F1 embryos) were maintained as mentioned above. To investigate whether the effect of parental hypermethioninaemia has a transgenerational effect in offspring, we evaluated a wide variety of experiments comprising protocols for morphology, heart rate, locomotor, behavioral, cognitive, and neurochemical analysis in larval (F1 larvae Met or control groups) and adult (F1 adult Met or control groups) zebrafish offspring. The study design is outlined in Fig. 1.

Larval analyses

Egg count and survival analyses

The egg count was performed in a Petri dish with water, using a Pasteur pipette on a black background. The results were obtained through six reproductions. For survival analysis, a total of 260 larvae (F1 larvae control group: 130; F1 larvae Met group: 130) were monitored for 12 days daily. Two individual analyses were performed, with two dishes per group.

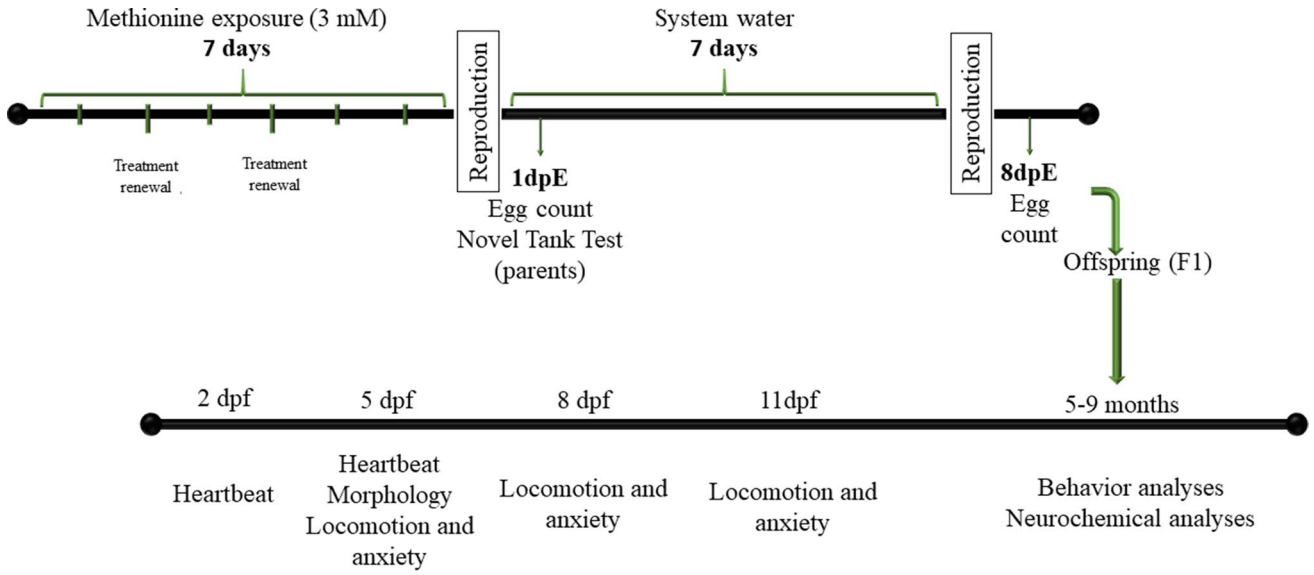

Fig. 1 Schematic summary of the experimental study design. The eggs were obtained from Methionine-treated and control groups and we evaluated the morphological, biochemical, and behavioral parameters at larvae and adult stage

Heart rate analysis

The F1 larvae were analyzed for their heart rate. This measurement was carried out at 48 and 120 h post-fertilization (hpf) using a stereomicroscope with a total of 67 animals (F1 larvae control group: 34; F1 larvae Met group: 33) in an experimental duplicate. The heart rates were monitored for 60 s in F1 larvae Met and control placed individually in well plates (Macchi et al. 2018). A thermal plate coupled to the stereomicroscope kept the temperature stable at 28 °C. To avoid bias, it was performed blind analyzes.

Morphological analyses

The F1 larvae at 5 dpf were analyzed in a stereomicroscope (3 × magnification) to evaluate morphological defects. A total of 67 animals were used (F1 larvae control group: 34; F1 larvae Met group: 33) with an experimental duplicate. Body length (µm), the distance from the larvae mouth to the pigmented tip of the tail was measure as described by Capiotti et al. (Capiotti et al. 2011) with modifications; the ocular distance (µm) was evaluated by the distance between the inner edge of the two eyes; the size of the eyes (μm^2) was determined by measuring the surface area of the eyes (Lutte et al. 2015). We used the NIS-Elements D software for Windows 3.2 (Nikon Instruments Inc, Melville, USA) to measure the parameters. To avoid bias, it was performed blind analyzes.

Exploratory behavior

We evaluated the exploratory behavior of the F1 larvae at 5, 8, and 11 dpf as previously described by Altenhofen et al. (Altenhofen et al. 2017b). A total of 59 animals was used (F1 larvae control group: 32; F1 larvae Met group: 27) in experimental duplicate. The apparatus was mounted in a temperature-controlled room (27 °C ± 2 °C) and the experiment was done between 13:00 h and 17:00 h. For each trial, a single larva was placed in a 24-well plate that contained 2.5 mL of water per well. After 1 min of acclimatization a 5 min session of exploratory behavior analysis was performed (Colwill and Creton 2011). The EthoVision XT software (version 11.5, Noldus) was used for the automated analysis of the recorded video. To evaluate the exploration of the new environment we consider the total distance traveled (m) and velocity (m/s, the ratio between distance traveled and movement) as parameters of locomotion. The time spent outside the area (s) and frequency of center entries were determined for evaluation of behavior related to anxiety/fear. The parameter movement was defined as the period in which the zebrafish exceeded the start velocity defined as 0.06 cm/s and remained moving until the stop velocity defined at 0.01 cm/s.

Adult analyses

Novel tank test

The novel tank test was performed to evaluate the anxiety-like behavior and locomotion as described previously by Gerlai et al. with modifications (Gerlai et al. 2000; Zanandrea et al. 2018). This experiment was performed in F1 adults and their parents 1dpE. In these experiments, 64 F1 adult animals were used (F1 adult control group: 31; F1 adult Met group: 33) and 69 animals as parents were used (Parent control group: 32; Parent control group: 37) in experimental triplicate. The animals were placed individually in an experimental tank (30 cm long × 15 cm high × 10 cm wide) and after 1 min of habituation were filmed for 5 min. The aquarium was virtually divided into consisted in two zones and the videos were analyzed with EthoVision XT software. The parameters analyzed were time spent in the upper zone(s), latency to upper zone(s), distance moved (m), and velocity (m/s). To prevent isolation from causing stress, no animals were left alone in the exposure tank. The experiment was conducted in a temperature-controlled room (27 ± 2 °C) between 09:30 h and 12:00 h.

Social interaction

The preference for living in groups is an innate behavior of the zebrafish; thus, the decrease in social interaction can represent behavioral disorders (Gerlai et al. 2000). The F1 adult zebrafish was submitted to this experiment. A total of 71 animals (F1 adult control group: 39; F1 adult Met group: 32) were tested in experimental triplicate. The apparatus consisted of an experimental tank (30 cm long × 15 cm high × 10 cm wide) where each fish was individually placed. Next to the test aquarium, there was an equal aquarium with only water and on the other side an aquarium with 15 zebrafish. The zebrafish behavior was recorded for 5 min after acclimatization of 60 s in the experimental tank. The recorded videos were analyzed by the EthoVision XT software, as described by Gerlai et al. (Gerlai et al. 2000) with modifications (Zanandrea et al. 2020). The tank was virtually divided into three zones to quantify fish preference between the "stimulus fish" side and the empty tank. The amount of time the experimental fish spent closer to the stimulus zone was measured. Social interaction was evaluated between 09:30 h and 12.00 h.

Mirror-induced aggression

Zebrafish are social animals: they exhibit social behavior as well as territoriality and aggression. The Mirror-induced aggression assay was used to measure aggression in F1 adults as described by Gerlai et al. (2000), with modifications. A

total of 71 animals (F1 adult control group: 39; F1 adult Met group: 32) were tested in experimental triplicate. The apparatus consisted of an experimental tank (30 cm long × 15 cm high × 10 cm wide) with a mirror (45 cm × 38 cm) placed at an angle of 22.5° to the back wall of the tank. The left vertical edge of the mirror touched the side of the tank while the right edge was farther away. Thus, the image of the experimental fish reflected in the mirror was closer as it swam to the left side of the tank. The fish remained in the aquarium for 6 min, the first minute for acclimatization and the other five minutes for recording their behavior. The analysis of recorded videos was done with EthoVision XT. To assess the position of the fish in each place of the tank, we divided the tank into six with three virtual vertical lines and one horizontal line. As aggression parameters, we measured the amount of time the experimental fish spent in the segment closest to the mirror and the number of bites against the mirror image. To avoid any bias, a blind analysis was performed by the evaluator in the bite parameter. Aggression behavior was evaluated between 9:30 h and 12:00 h.

Inhibitory avoidance task

We performed the inhibitory avoidance task in F1 adults to evaluate aversive memory (Blank et al. 2009; Nabinger et al. 2018). For this test, a total of 25 animals was used (F1 adult control group: 12; F1 adult Met group: 13) in experimental duplicate. The experimental apparatus of this test (18 cm long × 9 cm wide × 7 cm high) containing a door dividing the tank into two compartments of the same size, one white and the other black. The animals were placed individually in this apparatus to carry out a training session and a test session with an interval of 24 h between them. Initially, in the training session, the animals acclimatized for 1 min in the white compartment with the door closed. After this period, the door was carefully opened and when the fish passed to the black zone, the door was closed, and the animal carried a shock pulse of 3 ± 0.2 V AC for a period of 5 s. The intensity of shock pulse was measured between the two electrodes connected to an 8 V stimulator and in the center of the black compartment. After 24 h the animals passed the same protocol but without the electric shock. The parameter of memory is the latency to enter the black compartment during each session. The increase in latency was used as an index of memory retention. A 180 s ceiling was imposed on test session latency measurements. This experiment was carried out between 9:00 h and 12:00 h.

Biochemical parameters

Oxidative stress parameters

Tissue preparation The F1 adult zebrafish were euthanized by hypothermal shock, and the brains were removed and maintained in ice. Subsequently, five brains of fish generated by two reproductions ($n = 6$) were homogenized in 20 mM sodium phosphate buffer (pH 7.4), with 140 mM KCl. and centrifuged at 800*g* for 10 min at 4 °C. The supernatant was used for the analysis of oxidative stress parameters.

2′,7′-Dihydrodichlorofluorescein (H_2DCF) oxidation assay In this experiment, the molecule 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) is cleaved by esterases generating 2′,7′-dichlorofluorescein (H_2DCF), the H_2DCF is oxidized by ROS present in samples producing a fluorescent compound: dichlorofluorescein (DCF), measured at 488 nm excitation and 525 nm emission. ROS production was measured as described by LeBel et al. (1990). The results are presented as nanomoles of DCF per milligram of protein.

Thiobarbituric acid reactive substances (TBARS) TBARS measures malondialdehyde (MDA), a product of lipoperoxidation. TBARS were determined in a spectrophotometer by the absorbance at 535 nm (Ohkawa et al. 1979). Calibration curve was made using 1,1,3,3-tetramethoxypropane. The results are presented as nanomoles of malonaldehyde per milligram of protein.

Nitrite levels Nitrite is a metabolite of nitric oxide (NO) which was measured according to Greeb et al. (1982). The samples were mixed with Griess reagent to form a rosy product that was measured on a microplate reader (SpectraMax M5/M5 Microplate Reader; Molecular Devices, MDS Analytical Technologies) at 543 nm. The nitrite concentration was calculated using a sodium nitrite standard.

Superoxide dismutase (SOD) activity assay Superoxide anion radicals ($O_2.-$) derived from extracellular stimulants can be eliminated by the SOD enzyme that converts them to H_2O_2 (Miao and St. Clair 2009). SOD (EC 1.15.1.1) activity assay was accomplished according to Marklund and Marklund (Marklund and Marklund 1974). Pyrogallol is a self-oxidized compound in presence of superoxide. In the presence of the SOD enzyme, this reaction is inhibited, and this activity was assayed at 420 nm. A calibration curve was made with purified SOD as a standard. The results are presented as units per milligram of protein.

Catalase (CAT) activity assay CAT is an enzyme specialized in the quick elimination of H_2O_2. CAT (EC 1.11.1.6) activity was assayed based on the consumption of H_2O_2 at 240 nm according to Aebi (1984). The samples were mixed with a 0.06% solution of H_2O_2, and its consumption was measured every 30 s for 5 min in a spectrophotometer. The results are presented as units per milligram of protein, where 1 CAT unit is 1 µmol of H_2O_2 consumed per minute.

Determination of neurotransmitter and amino acids by liquid chromatography–tandem mass spectrometry (LC–MS/MS)

We analyzed the neurotransmitters and amino acids in the F1 adult zebrafish brain. The samples were prepared as described by previous studies by our group (Altenhofen et al. 2017b; Zanandrea et al. 2020). Briefly, five brains of fish generated by two reproductions were homogenized in 500 µL of 0.1 M formic acid (Sigma-Aldrich, St. Louis, MO) and centrifuged at 20,000*g* for 20 min at 4 °C. The supernatant was transferred to a 250 µL glass vial and injected into an ultra-high performance liquid chromatography, coupled to a mass spectrometer, (Agilent, Santa Clara, CA, USA). Chromatographic separations were performed on a Zorbax Eclipse Plus C18 RRHD column, 5 × 2.1 mm, 1.8 µm (Agilent, Paolo Alto, USA) using 2 mobile phases (A, 0.1%formic acid and B, acetonitrile with 0.1% formic acid). Quantifications were performed by external standardization. Standards were prepared individually in the water at a concentration of 0.5 mg/mL. Before the analyses, they were mixed and diluted with mobile phase A and were added with beta-mercaptoethanol in an equivalent concentration of the samples. The results were corrected by the protein concentration of the samples.

Protein determination

To normalize both amino acid concentration and neurotransmitter, the protein content of the samples was determined as described for Bradford (1976), and the oxidative stress parameters according to Lowry et al. (1994). Serum bovine albumin was used as a standard for the calibration curve.

Statistical analysis

Two-way analysis of variance (ANOVA), followed by Tukey's test was used to compare differences between egg production of parents exposed to Met. The Kaplan–Meier analysis was performed to compare larval survival during 12 experimental days. Welch's *t* test was used to compare the data from exploratory behavior, novel tank test, social interaction, mirror-induced aggression, oxidative stress parameters, amino acid, and neurotransmitter analyses. Mann–Whitney *U* tests compared the data of inhibitory avoidance memory task. All data are expressed as the mean ± standard error (S.E.M). For all comparisons, the significance level was set at $p < 0.05$.

Results

Met levels in water tanks

To determine the Met levels present in the treatment tanks, we collected the samples before and after preparing the waters for the treatment of the animals. After analysis by LC–MS/MS, we found a mean of 2.743 ± 0.127 mM (as standard deviation) for the Met group treatment tank and it was not detected for the control group.

Egg count and survival of larvae offspring

The exposure of parents to 3 mM Met caused an acute effect on zebrafish fertility: egg production was strongly reduced ($p = 0.0391$) at 1 day post exposure (dpE), [Treatment: $F_{(1,18)} = 9.577$, $p = 0.0063$; Time: $F_{(1,18)} = 3.972$, $p = 0.0616$; Interaction: $F_{(1,18)} = 1.593$, $p = 0.2230$]. The egg count returned to normal values at 8 dpE ($p = 0.5394$) (Fig. 2a). To evaluate whether Met exposure affects the offspring survival, we monitored the animals from 0 to 12 dpf. The survival analyses did not show any significant alteration (Fig. 2b, $p = 0.0640$).

Morphological analyses and heart rate in larval offspring

To better understand the potential effects of Met on offspring development, we performed morphological analyses at 5 dpf and the analysis of heart rate at 48 and 120 hpf. There were no significant differences in body length (Fig. 3a, $p = 0.0676$), ocular distance (Fig. 3b, $p = 0.1516$), ocular surface area (Fig. 3c, $p = 0.1263$) or heart rate (Fig. 3d) at 48 hpf ($p = 0.0632$) and 120 hpf ($p = 0.5926$) between the F1 larvae control group and F1 larvae Met group.

Exploratory behavior of larval offspring

To determine whether Met exposure in parents can alter locomotion and anxiety behavior in offspring at the larval stage, we assessed the exploratory behavior at 5, 8, and 11 dpf. There was an increase in distance traveled in the F1 Met larvae at 5 dpf ($p = 0.0205$) and 8 dpf ($p = 0.0278$) (Fig. 4a) as well as an increase in mean speed at 5 dpf ($p = 0.0125$), 8 dpf ($p = 0.0329$), and 11 dpf ($p = 0.0040$) (Fig. 4b). The frequency in center zone (Fig. 4c) in 5 dpf ($p = 0.0574$), 8 dpf ($p = 0.1841$) and 11 dpf ($p = 0.4851$) and time spent at the center (Fig. 4d) in 5 dpf ($p = 0.6405$), 8 dpf ($p = 0.0755$), and 11 dpf ($p = 0.5246$) did not change significantly between de groups.

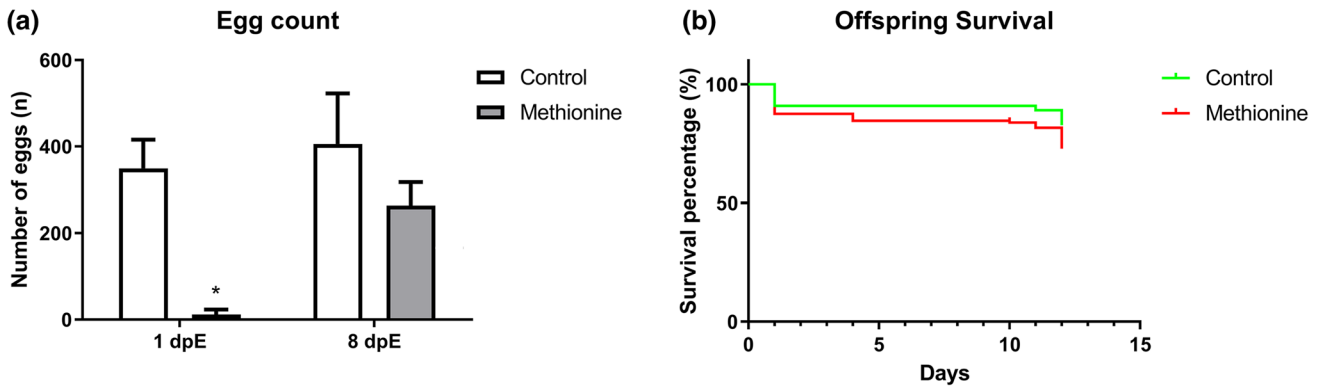

Fig. 2 Egg count and survival analyze. **a** Egg account in 1 day post exposure (dpE) and 8 dpE to 3 mM Met. The animals were exposed to 3 mM of L-Mehionine (Methionine) or water (Control) for 7 days. Statistical analysis was performed by two-way ANOVA followed by Tukey test, the data are expressed as mean ± S.E.M. Results were obtained from 5 to 6 reproductions. **b** Kaplan–Meier survival for F1 larvae, statistical analysis was performed by Log-rank (Mantel–Cox) test. Data are expressed as the mean ± S.E.M from 35 animals, in quadruplicate, analyzed individually for each group. Data are expressed as the mean ± S.E.M. Statistical analysis was performed by Welch's *t* test, (*$p < 0.05$)

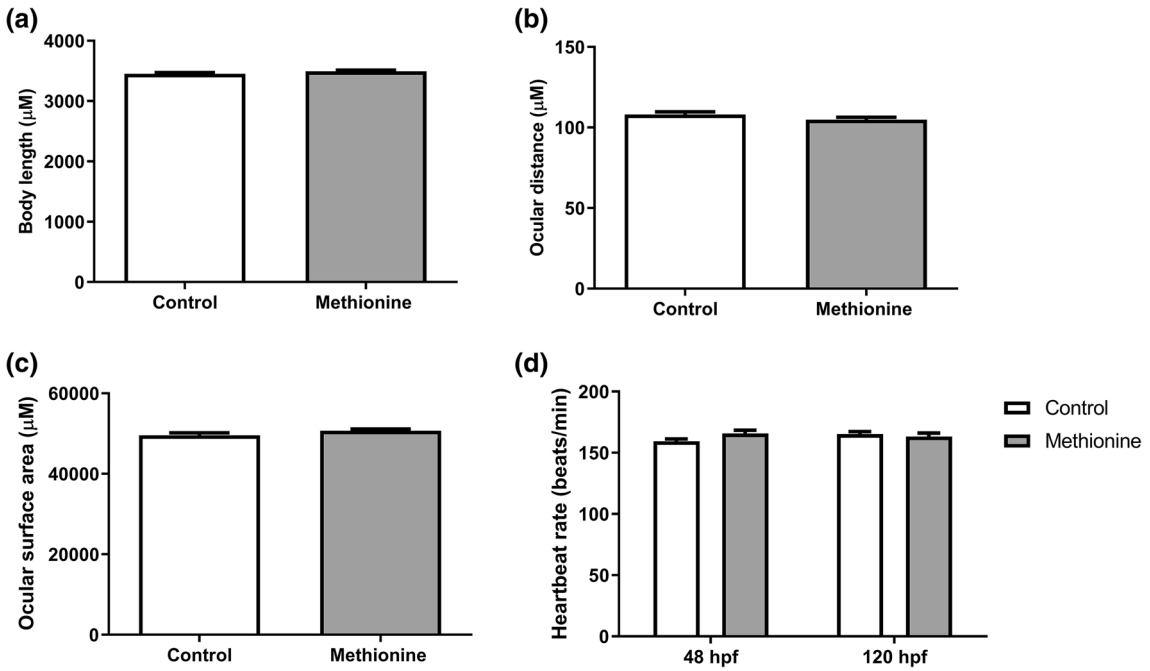

Fig. 3 Morphological parameters and heartbeat rate of F1 larvae control (Control) and F1 larvae Methionine (Methionine) groups. **a** Body length; **b** Ocular distance, and **c** Ocular surface area measured at 120 h post-fertilization (hpf) and **d** Heartbeat rate measured at 48 and 120 hpf. Data are shown as the mean ± S.E.M., with two individual experiments. Statistical analyses were performed by Welch's *t* test

Novel tank test

The Novel Tank Test was performed in adult zebrafish offspring. Locomotion parameters (distance and mean speed) of the F1 adult Met group did not change significantly when compared to the F1 adult control group (Fig. 5a, $p = 0.6242$; 5b, $p = 0.4700$). There was a significant increase in the time spent in the upper zone of the F1 adult Met group when compared to the F1 adult control group (Fig. 5d, $p = 0.0258$), but there were no significant differences in latency to the upper zone (Fig. 5c, $p = 0.0841$).

The Novel Tank Test was also performed in parents at 1 dpE. There was a significant decrease in mean speed (Supplementary Fig. 1b, $p = 0.0057$), and latency to the upper zone (Supplementary Fig. 1c, $p = 0.0005$), while the time in the upper zone increased (Supplementary Fig. 1d, $p < 0.0001$;). Distance was not altered in parents 1 dpE (Supplementary Fig. 1a, $p = 0.2621$).

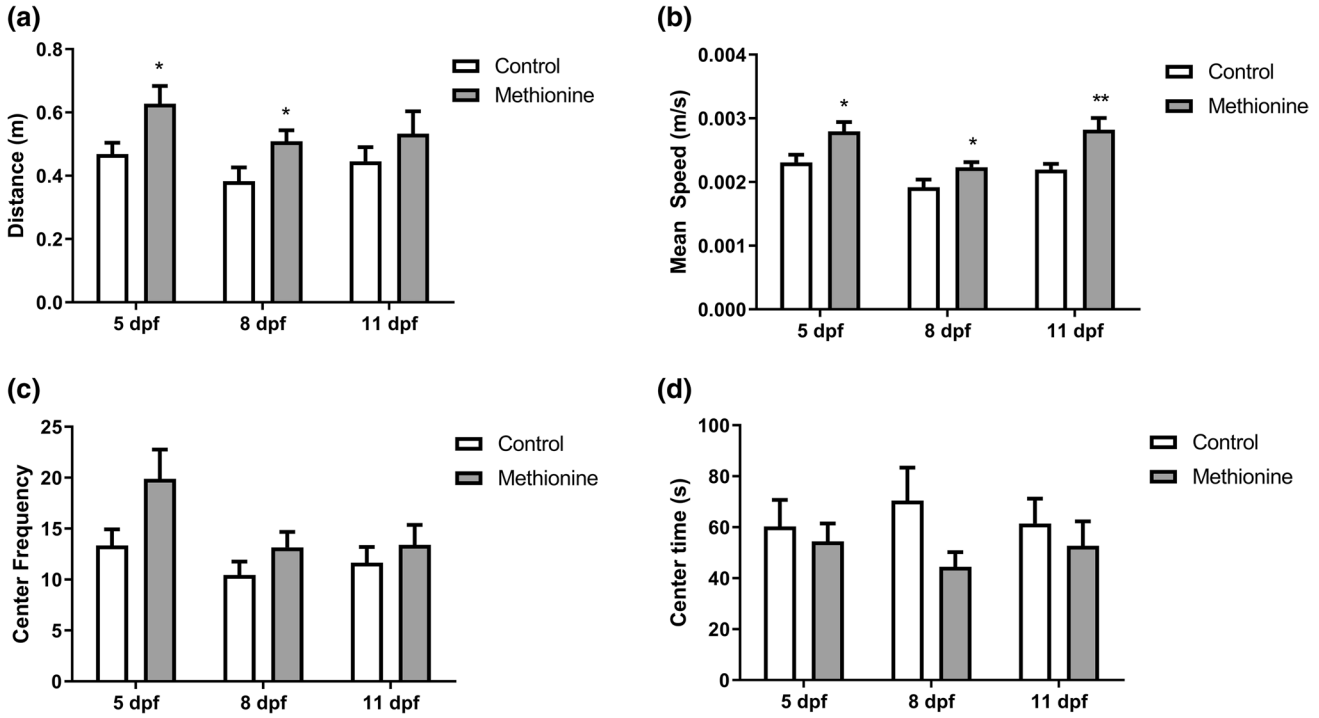

Fig. 4 Exploratory behavior in F1 larvae control (Control) and F1 larvae Methionine (Methionine) groups at 5, 8, and 11 days post fertilization. **a** Total distance traveled, **b** Mean speed, **c** Frequency in center zone and **d** Time spent in center zone. Data are expressed as the mean ± S.E.M. Statistical analyses were performed by Welch's *t* test, (*$p < 0.05$; **$p < 0.01$)

Aggression and social interaction

The aggressive behavior evaluated by the time spent in the segment nearest to the mirror ($p = 0.3286$) and the number of bites ($p = 0.1824$) in this zone did not show differences between the F1 adult Met group and the F1 adult control group (Fig. 6a, b). Furthermore, the Met exposure did not induce any social interaction deficit in zebrafish offspring; all animals presented the same preference for the stimulus area as their respective controls (Fig. 6c, $p = 0.1891$).

Inhibitory avoidance task

Long-term memory (inhibitory avoidance task) was analyzed in F1 adult zebrafish. The significant differences in the latency for the training and test sessions in the F1 adult Met group demonstrated that there was no impairment in aversive memory (Fig. 7, $p < 0.0001$). The respective control group also demonstrated a significant difference between training and testing sessions (Fig. 7, $p < 0.0001$).

Oxidative stress parameters

The oxidative stress parameters were evaluated in zebrafish offspring brains. We found significant increases in DCFH oxidation (Fig. 8a, $p = 0.0415$) in the F1 adult Met group, when compared to the respective control group. On the other hand, TBARS (Fig. 8b, $p = 0.2677$), Nitrites (Fig. 8c, $p = 0.4850$), SOD activity (Fig. 8d, $p = 0.0828$), CAT activity (Fig. 8e, $p = 0.7547$), and the ratio SOD/CAT (Fig. 8f, $p = 0.0564$) did not change significantly when compared to control.

Neurotransmitter and amino acid analyses

The effect of parental exposure to Met on amino acids and neurotransmitters was evaluated in the brains of zebrafish offspring. Dopamine (Fig. 9a, $p = 0.0093$), serotonin (Fig. 9b, $p = 0.0238$), and glutamate (Fig. 9d, $p = 0.0025$) levels increased significantly, while epinephrine did not change (Fig. 9c, $p = 0.0779$). Increased glutathione levels were also observed in the F1 adult Met group (Fig. 9g, $p = 0.0207$). There were no significant changes in Met (Fig. 9e, $p = 0.2690$), homocysteine (Fig. 9f, $p = 0.5628$), carnitine (Fig. 9h, $p = 0.2146$), creatine (Fig. 9i, $p = 0.0654$), taurine (Fig. 9j, $p = 0.6220$), or cysteine levels (Fig. 9k, $p = 0.3562$).

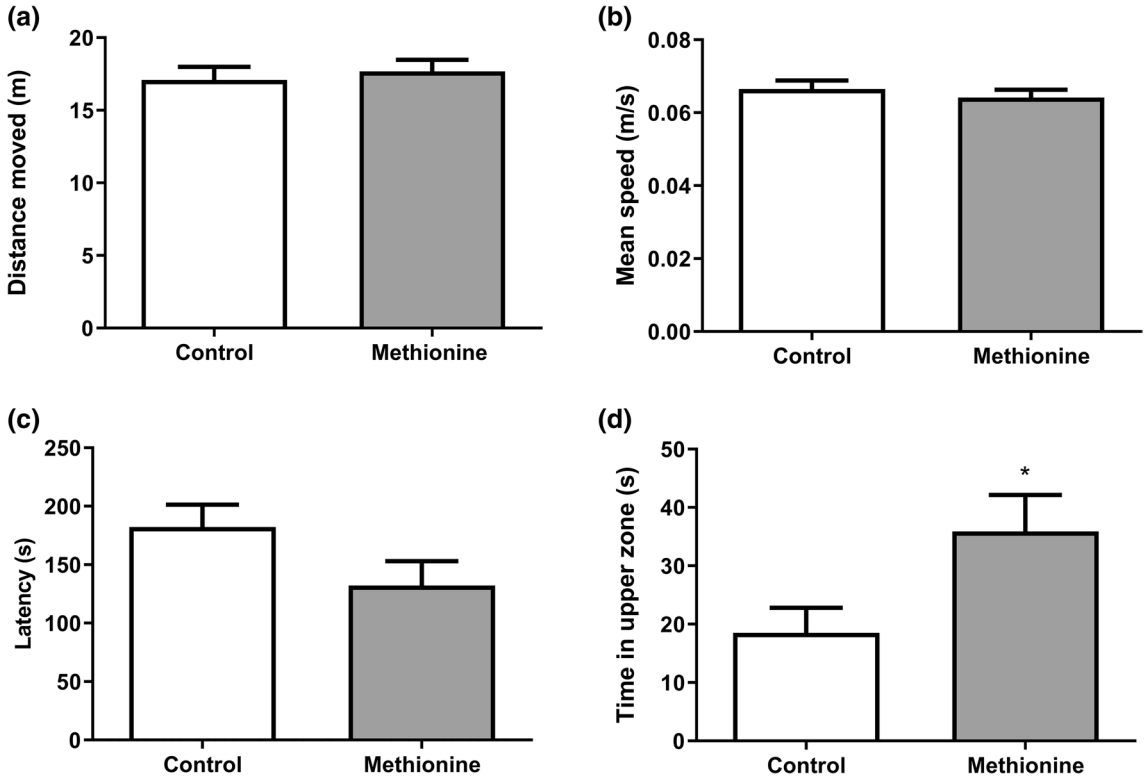

Fig. 5 Novel Tank Test parameters in F1 adult control (Control) and F1 adult Methionine (Methionine) groups. **a** Distance moved; **b** Mean speed; **c** Latency to enter in upper zone; **d** Time in upper zone. Data are expressed as mean ± S.E.M. Statistical analyses were performed by Welch's *t* test, (*$p < 0.05$)

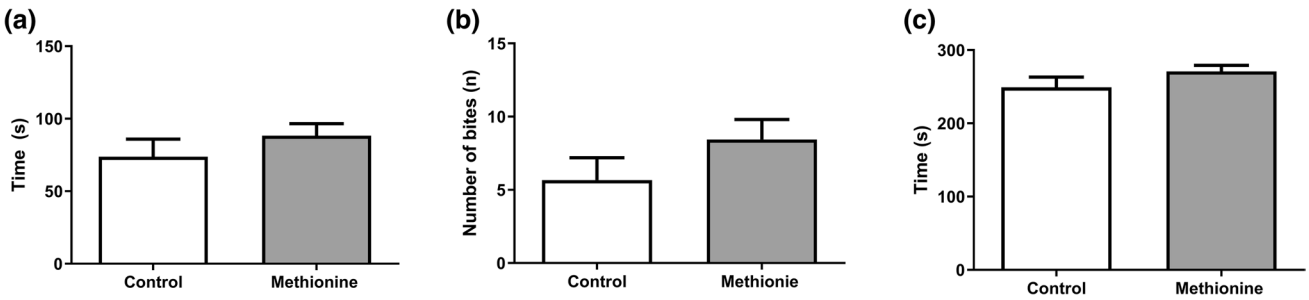

Fig. 6 Social interaction test and aggression parameters in F1 adult control (Control) and F1 adult Methionine (Methionine) groups. **a** Time in mirror zone; **b** Number of bites; **c** Time in interaction zone. The data are expressed as the mean ± S.E.M. and were analyzed by Welch's *t* test

Discussion

In the present study, we examined whether a transient excessive Met exposure in zebrafish before mating exerts transgenerational effects in the offspring at the larval and adult stage. We observed changes in locomotion in the F1 larvae Met group and alterations in anxiety-like behavior in the F1 adult Met group associated with increases in ROS and imbalance in the dopamine, serotonin, and glutamate levels. The neurophysiological changes, previously seen in the hypermethioninaemic zebrafish (Vuaden et al. 2012, 2016; Zanandrea et al. 2020), were also observed in the offspring, suggesting a transgenerational effect of Met.

Fetal development requires homeostasis in the Met cycle (Rees et al. 2006). The excess of homocysteine, a metabolite of Met, has a positive correlation with pregnancy complications, such as early spontaneous abortions and fetus death (Gaiday et al. 2018). Our data demonstrated that the excess Met is detrimental to fertility, given that the exposed animals showed a marked decrease in egg production at 1 dpE. The mechanism underlying this effect is not the focus of our

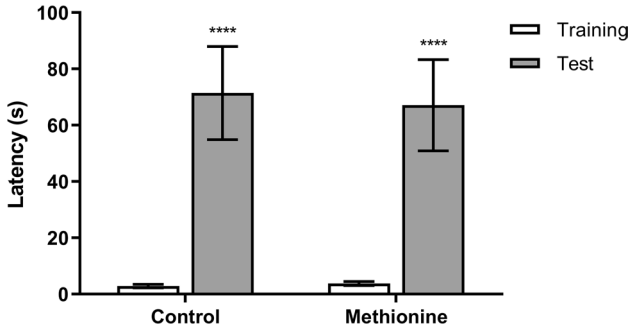

Fig. 7 Inhibitory avoidance task performance on training and long-term memory test sessions in F1 adult control (Control) and F1 adult Methionine (Methionine) groups. The data are expressed as the mean ± S.E.M. and were analyzed by Welch's *t* test (****indicates $p < 0.0001$ when compared to training session)

study; however, the Novel Tank Test performed in parents exposed to Met at 1 dpE suggests that increased anxiety was not responsible for the impaired egg production (Supplementary Fig. 1). Parents exposed to high Met concentration at 1dpE showed a decrease in anxiety-like behavior seen through the increased time in the top zone, decreased mean speed, and decreased latency time to enter in top zone. Studies conducted by our group with parents after 8 dpE to Met also showed a decrease in anxiety-like behavior, which together with other behavioral changes showed a neurotoxic effect of Met (Zanandrea et al. 2020). Few studies have addressed the mechanisms by which excess Met causes infertility. Inadequate ovarian steroid secretion as estrone and progesterone caused by Met reduces pregnancy maintenance in rats (Chandrashekar and Leathem 1977). Another possible mechanism involves an excess of homocysteine: high levels are associated with lower pregnancy rates in women (Ocal et al. 2012). Our study demonstrated that the egg production returned to values close to normal at 8 dpE. Our group observed that the homocysteine levels were normalized 8 days after the withdrawal of Met (Zanandrea et al. 2020), a finding that can explain the fertility recovery. Furthermore, there were no significant differences in the parameters of larvae length, ocular distance, and eye size at 5 dpf. The cardiac function evaluated at 2 and 5 dpf was also preserved. Consequently, larval survival was similar in both groups. These impairments may only occur when the Met/homocysteine levels are high in parents and a transient increase has not a long-lasting effect on parental infertility and offspring morphology.

The excessive Met parental exposure caused an increase in distance moved at 5 and 8 dpf and velocity at 5, 8, and 11 dpf in the F1 larvae. However, in the F1 Met adult group, our data showed that locomotion was not altered, as previously reported (Ryan et al. 2018; Schweinberger et al. 2018). There were no differences between the total distance and the mean speed between the F1 larvae Met and control groups. These findings suggest that excess Met exposure in parents can cause hyperactivity during the early stages of offspring development, but this effect disappears in adulthood. On the other hand, the reduced anxiety observed in Met-exposed adult zebrafish (Zanandrea et al. 2020) is passed on to the offspring, since the time in the upper zone for the F1 adult Met group was significantly higher compared to the control group. Therefore, these results suggest that parental high Met concentration exposure impacts the behavioral phenotypes of the offspring in larval and adult stages.

Gestational hypermethioninaemia in rats decreases the number of neurons and is associated with a decrease in nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) levels in the offspring brain (Schweinberger et al. 2017). These changes can be predisposing factors for the development of behavioral changes observed in the offspring. Gestational hypermethioninemia of rats reduced exploration and cause memory impairment in offspring (Schweinberger et al. 2018). Our results did not show significant differences between the F1 adult Met and control groups regarding social interaction and aggressive behavior. We also did not observe changes in aversive memory. A diet rich in methyl donors of parent rats showed that long-term potentiation (LTP) in the offspring hippocampus is reduced; however, some aspects of synaptic function are not affected (Ryan et al. 2018). This outcome could explain why some behavioral parameters are unchanged. We, therefore, suggest that transient high hypermethioninemia in parents may affect specific offspring behaviors, such as anxiety-like behavior.

To best understand the biochemical and neurochemical mechanisms underlying the behavioral effects observed in the F1 adult Met group, we performed the analysis of amino acids involved with the Met cycle and oxidative stress parameters. Voltage- and Ca_2^+-activated K^+ (BK) channels are expressed in neuronal tissues; they regulate neurotransmitter release and neuronal excitability. Consequently, they are involved in learning and memory (Raffaelli et al. 2004; Lee and Cui 2010). The BK channel beta subunit 2, encoded by *Kcnmb2*, plays a role in the inactivation of BK channels (Yu et al. 2018), and methyl donors have a transgenerational effect, specifically by altering *Kcnmb2* methylation, a phenomenon that decreases its expression (Ryan et al. 2018). These changes may be involved with our findings, where some neurotransmitters are increased. Parental exposure to excessive Met produced transgenerational effects on dopamine, serotonin, and glutamate levels, namely increasing these neurotransmitters in the F1 adult Met group. Neuronal hyperexcitability is observed in brain lesions and it promotes changes in gene regulation by altering the balance of channels, receptors, neurotransmitters, or transporters (Penas and Navarro 2018). Abnormalities in neurotransmission indicate an interruption in the processing of neuronal information, a

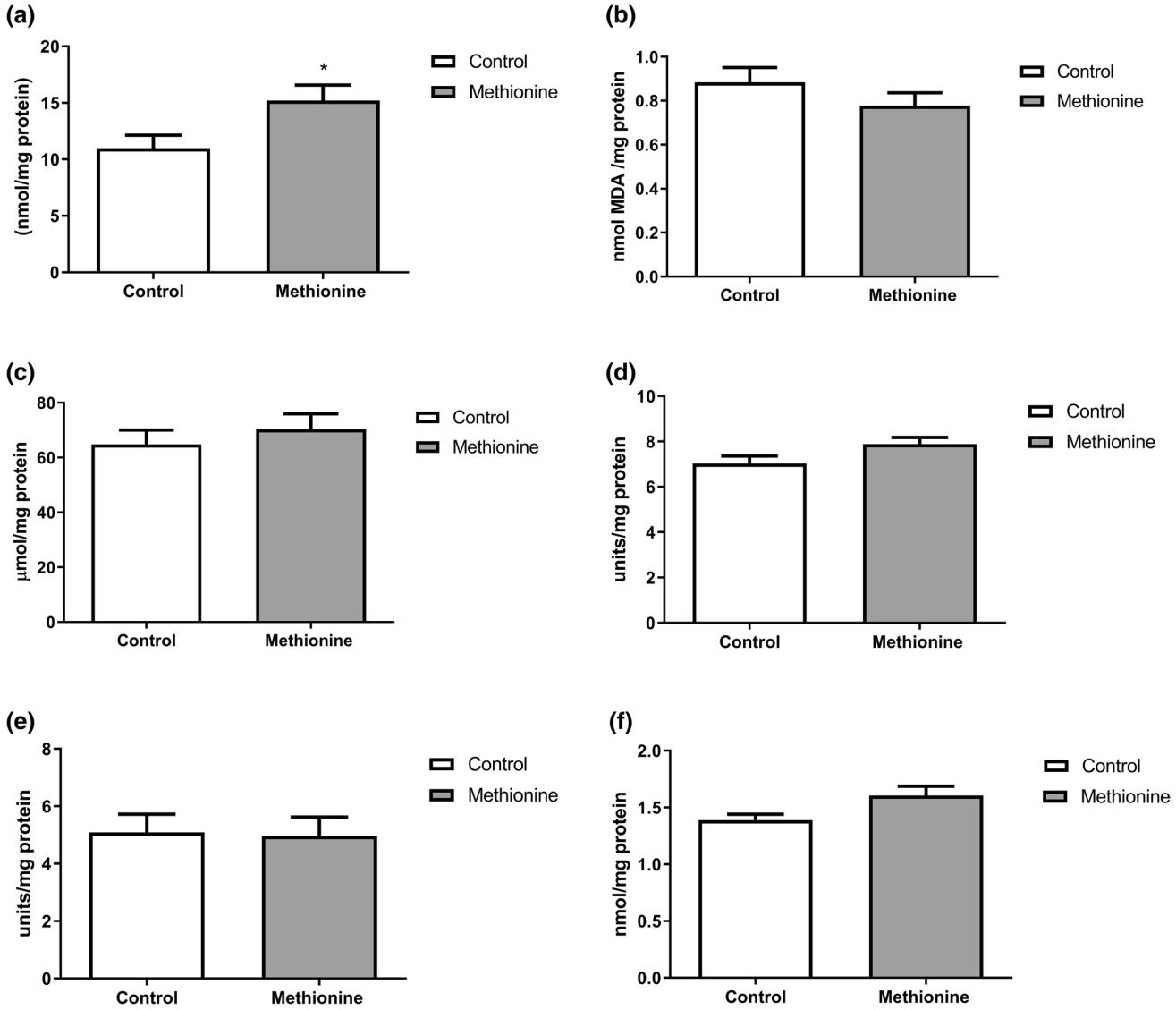

Fig. 8 Oxidative stress parameters in F1 adult control (Control) and F1 adult Met (Methionine) groups. **a** DCFH oxidation; **b** TBARS; **c** Nitrites; **d** SOD activity; **e** CAT activity; and **f** Ratio SOD/CAT in zebrafish brain. Data from DCFH oxidation are expressed as nmol of DCF formed per mg of protein; data from TBARS are expressed as nmol of TBARS formed per mg of protein; Enzyme activities are expressed as units per mg of protein. One CAT unit is defined as 1 µmol of oxygen peroxide consumed per minute. One SOD unit is defined as the amount of SOD necessary to inhibit 50% of pyrogallol autoxidation. Data are expressed as mean ± SEM and were analyzed by Welch's *t* test (*$p < 0.05$)

phenomenon that can compromise cognitive functions and lead to behavioral dysfunctions (Sarter et al. 2007).

Dopamine is well known for having important effects on behavior modulation and complex cognitive functions, such as reasoning, language comprehension, planning, locomotion, and spatial processing (Cools and D'Esposito 2011; Steinberg and Janak 2013). The projections of the dopaminergic system in the brain are very broad, including the prefrontal cortex and amygdala, in which the dopaminergic system is affected clearly, inducing different cognitive and behavioral effects (Steinberg and Janak 2013). Dysfunction in this system might be implicated in the pathogenesis of social anxiety in patients with Parkinson's disease, depression, and schizophrenia (Moriyama et al. 2011; Steinberg and Janak 2013; Brisch et al. 2014). Glutamatergic alterations also play an important role in the pathophysiology of schizophrenia (Legind et al. 2019), and blood and brain glutamate levels were significantly higher in patients with autism (Hassan et al. 2013). Serotonin acts ubiquitously on a variety of receptors and abnormalities in an animal model of serotonin syndrome showed an increase in glutamate and dopamine levels. Dopamine levels, as well as serotonin

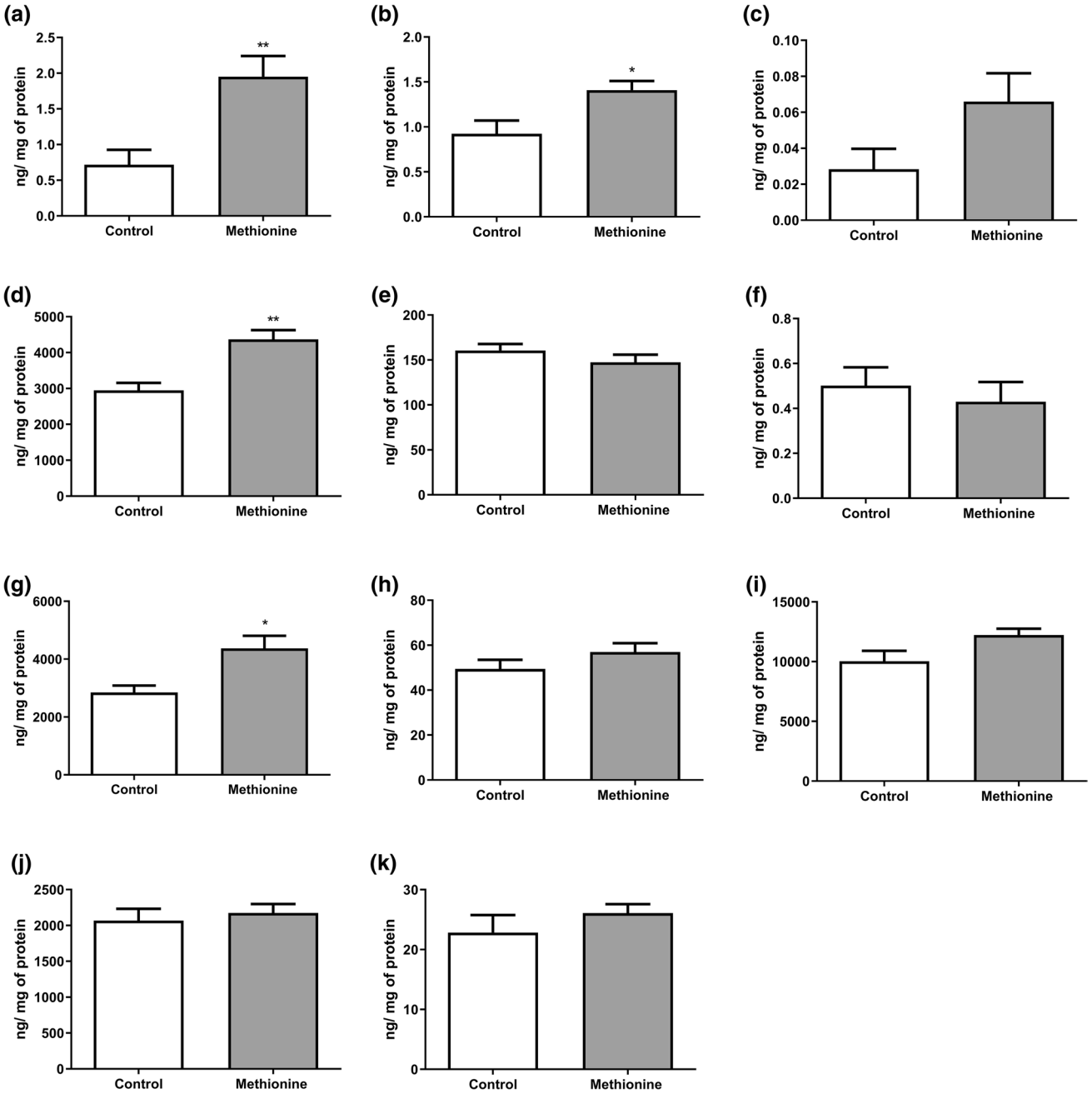

Fig. 9 Neurotransmitters and amino acids analyze in F1 adult control (Control) and F1 adult Met (Methionine) groups. **a** Dopamine; **b** Serotonin; **c** Epinephrine; **d** Glutamate; **e** Methionine; **f** Homocysteine; **g** Glutathione; **g** Carnitine; **i** Creatine; **j** Taurine; **k** Cysteine. The parameters are expressed as ng/mg of protein and data are expressed as mean ± SEM and analyzed by Welch's *t* test (*$p < 0.05$ and ** to $p < 0.01$)

levels, increased markedly before glutamate levels increased, suggesting dopamine involvement in the control of glutamate levels (Shioda et al. 2004). A plausible hypothesis for the increase in dopamine and serotonin levels observed in the F1 Met adult group may be related to a modulation of the enzymes involved in the catabolism of monoamines. Therefore, the evaluation of these enzymes in the offspring after parental exposure to excessive Met seems interesting, especially the Monoamine oxidase (MAO) and Catechol O-methyltransferase (COMT).

Prolonged or excessive exposure to the excitatory transmitter glutamate harms neurons, and this effect can be mediated by the generation of ROS (Rao et al. 2003). Thus, the increased glutamate levels seen in our study can induce higher ROS production, as observed in the F1 adult Met group. The increase in ROS can contribute to the oxidation

of proteins and thus decrease the content of total thiols, already evaluated in the offspring of hypermethioninaemic rats (Schweinberger et al. 2014). Our findings of TBARS levels and SOD also agree with Schweinberger et al. (2014), where gestational hypermethioninaemia did not alter these parameters (Schweinberger et al. 2014). High Met levels are responsible for decreasing CAT activity in the cerebral cortex of rats (Soares et al. 2017). Furthermore, only higher doses of Met administered to pregnant rats increased Met in the offspring brain; they also decreased CAT activity (Schweinberger et al. 2014). In our study, we did not observe significant changes in CAT activity, and this outcome might be due to the normal Met values found in the offspring brain.

In a model of gestational hypermethioninaemia in rats, Met levels are increased in the offspring brain (Schweinberger et al. 2014). However, in our study, there were no significant changes in the analyzed amino acids, including Met, between the F1 adult control group or the F1 adult Met group. In our model using zebrafish, the Met levels of offspring can be related to the transient hypermethioninaemia induced in the parents. While the Met levels are high in mother rats (Schweinberger et al. 2014), after 8 dpE, zebrafish parents do not appear to have elevated levels of Met or homocysteine (Zanandrea et al. 2020). Glutathione, a tripeptide of glutamate, cysteine, and glycine, in turn, showed high levels in Met offspring compared to the control, probably as a regulatory mechanism against the increase in ROS that is occurring.

Conclusion

Our study demonstrated that parental exposure to excessive Met, even if transient, had harmful effects: decreased egg production at 1dpE, increased locomotion F1 larvae Met group, and decreased anxiety-like behavior in F1 adult Met. In addition, there was an increase in DCF oxidation and changes in dopamine, serotonin, glutamate, and glutathione levels in the offspring from parents exposed to excessive Met. Given that transient hypermethioninaemia is often disregarded and the use of dietary supplements is growing in the population, a greater understanding of the influence of high levels of plasma Met is necessary to avoid behavioral and metabolic changes in subsequent generations. Taken together, our results show a transgenerational effect that affects the neurochemical homeostasis of the brain, leading to mental health issues and suggests the possibility that children's behavioral changes might be significantly influenced by the Met levels in parents.

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Authors' contributions Conceptualization: RZ, CDB; Data Curation: RZ; Methodology: RZ; Formal analysis and investigation: RZ, MTW, SA, GR, TMdS; Writing—original draft preparation: RZ; Writing—review and editing: RZ; ATSW; CDB; Funding acquisition: CDB; Resources: ATSW, CDB; Supervision: CDB.

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Availability of data and material Data will be made available on reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethics approval This study was approved by the Comissão de Ética no Uso de Animais of Pontifícia Universidade Católica do Rio Grande do Sul (3758-CEUA-PUCRS) and was registered in the Sistema Nacional de Gestão do Patrimônio Genético e Conhecimento Tradicional Associado—SISGEN (Protocol No. A3B073D).

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