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Original Article

The effects of isocaloric diets derived from different lipid sources on zebrafish

Os efeitos de dietas isocalóricas derivadas de diferentes fontes lipídicas em zebrafish

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Abstract

Characterizing the effects of saturated fat intake on metabolic health and its changes remains a major challenge. Lipid diets, from different sources, vary widely in their physiological effects on health; therefore, it is important to consider the specific lipid source consumed. The objective of the study was to evaluate the effect of the imposition of isocaloric diets with different lipid sources in zebrafish (fish oil/pork lard). Depicting how metabolic, morphological and behavioral parameters might express themselves in these fishes. Forty adult female fishes were used for the experiment. The animals were divided into a control group (C), fed with unsaturated fatty acid diet, and a saturated fatty acid group (Sat). They received food three times a day, during the 11-week period. The results showed that animals in the Sat group had increased body weight, with a difference relative to the C group, from the third week of diet until the end of the experiment. At the end of the last week, the Sat group had a body weight 32% higher (P=0.0182) than the body weight of the control group. The consumption of a diet rich in saturated fatty acids did not generate signs related to stress and anxiety in zebrafish. There was an increase in glycemia at T60 and T120, with a statistically significant difference between the two moments. Animals in the Sat group showed an increase (P=0.0086) in hepatic steatosis compared to animals in the control group. The results obtained on the relationship between diet and metabolic changes are fundamental to ensure the understanding and appropriate treatment of these problems.

Keywords: body mass, glucose, hepatic steatosis, fatty acids, metabolism.

Resumo

Caracterizar os efeitos da ingestão de gordura saturada na saúde metabólica e suas mudanças continua sendo um desafio importante. Dietas lipídicas, provenientes de diferentes fontes, variam amplamente em seus efeitos fisiológicos na saúde; portanto, é importante considerar a fonte lipídica específica consumida. O objetivo do estudo foi avaliar o efeito da imposição de dietas isocalóricas com diferentes fontes lipídicas (óleo de peixe/gordura suína) em zebrafish e descrever como parâmetros metabólicos, morfológicos e comportamentais podem se manifestar nesses peixes. Quarenta peixes fêmeas adultas foram utilizados para o experimento. Divididos em grupo Controle (C), alimentado com ração com ácido graxo insaturados e grupo ácido graxo saturado (Sat). Eles receberam comida três vezes ao dia, durante o período de 11 semanas. Os resultados mostraram que os animais no grupo Sat tiveram aumento de peso corporal, com diferença em relação ao grupo C, a partir da terceira semana da dieta até o final do experimento. No final da última semana, o grupo Sat teve um peso corporal 32% maior (P=0,0182) do que o peso do grupo controle. O consumo de uma dieta rica em ácidos graxos saturados não gerou sinais relacionados ao estresse e ansiedade em zebrafish. Houve um aumento na glicemia em T60 e T120, com diferença estatisticamente significativa entre os dois momentos. Animais no grupo Sat mostraram um aumento (P=0,0086) na esteatose hepática em comparação com animais no grupo controle. Os resultados obtidos sobre a relação entre a dieta e as alterações metabólicas são fundamentais para garantir a compreensão e o tratamento adequado desses problemas.

Palavras-chave: massa corporal, glicose, esteatose hepática, ácidos graxos, metabolismo.

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1. Introduction

The zebrafish is a promising vertebrate biological model for several health-related research fields (Zang et al., 2015). Despite the growing interest in *D. rerio* as a biological model, no standardized diets for their maintenance and growth have been established, as there are for rodents. As a result, it has become common practice for researchers to use commercially formulated diets for ornamental fish (Fowler et al., 2019) or some practical diets considered specie-specific (Fowler et al., 2021). However, there are scarce studies on zebrafish's nutritional requirements, which has hampered the development of balanced and nutritionally adequate practical diets (Clevenger et al., 2014).

For instance, optimal lipid content for other cyprinid species has been estimated to range between 6 to 9%. However, some commercial diets, including those considered species-specific, have been found to contain around 20% crude lipid (Fuente-Martín et al., 2013; Stubbins et al., 2012). Furthermore, the presence of anti-nutritional factors or feed additives in practical diets can introduce experimental bias that is difficult to measure in zebrafish studies, especially when measuring health or metabolic parameters. Therefore, it is crucial to carefully consider and control these factors when designing and conducting experiments in this model organism.

The use of semi-purified diets is recommended when the knowledge about nutritional requirements, digestibility, and inclusion levels of feedstuffs has not been established in diets for aquatic species. For the zebrafish, an additional aspect of using semi-purified diets is biosecurity. These diets minimize the probability of unknown mutritional aspects influencing the results of research. Semi-purified diets are formulated with ingredients with high biological value, which provide usually a specific nutrient such as protein, lipid and, carbohydrates. Consequently, research employing zebrafish as an alternative experimental model for rodents should use semi-purified diets as the preferred option until the premises for producing practical diets are established (Watts et al., 2016).

The effects of dietary lipid sources on physiological health are substantial, and interactions between fatty acids and lipid sources must be considered when using commercial diets for zebrafish due to the difficulty in isolating specific effects. In fact, only recently *D. rerio* has been used as an experimental model for metabolic diseases such as obesity (Anderson et al., 2011; Flynn III et al., 2009). Body mass gain, adiposity, and energy expenditure were influenced by the source and level of dietary lipids (Anderson et al., 2011; Flynn III et al., 2009).

This study aimed to assess the impact of semi-purified diets containing unsaturated (fish oil) and saturated (pork lard) lipid sources on metabolic, morphological, and behavioral parameters in female zebrafish. To this end, the animals were divided into groups: a control group (C) that received semipurified feed and a group that received saturated fatty acid (Sat).

2. Material and Methods

2.1. Animals and diets

The study was approved according to the Ethical Principles of Animal Experimentation, adopted by the National Council for Control of Animal Experimentation (CONCEA) and the Ethics Committee on Animal Use of Federal University of Paraná - Palotina (Protocol number 32/2020).

All 40 adult female fish were maintained in aquariums (4 fish/L) with temperature control (24 ± 2 °C) and suplementar aeration (> 5 mg/L). Ammonia (≤0.02 mg/L), nitrite (≤0.1 mg/L), nitrate (≤30mg/L), pH (7.0-7.5), and dissolved oxygen levels were monitored every day using a Labcon test (Alcon®, Santa Catarina, Brazil). The zebrafish (*D. rerio*) were housed in 3–L polycarbonate aquariums containing a water recirculation system under a 14 h light/10 h dark photoperiod.

The zebrafish were divided into two experimental groups of 20 fish (5 months post fertilization): the control group (C) receiving unsaturated fatty acids from fish oil and the saturated fatty acid group (Sat) feeding diets with pork lard. The semi-purified diets were nutritionally similar except for lipid sources (Table 1). Diets were provided thrice daily (8:00, 12:00, and 16:00) until apparent satiety over 11 weeks (Figure 1).

2.2. Experimental procedures and sampling

Body weigth and total body length were evaluated weekly after a 16-hour overnigth fast. The body mass

Table 1. Ingredients and chemical composition (wet basis) of control (fish oil) and saturated (pork lard) semi purified diets.

Ingredients (g/kg)	Control (C)	Saturated (Sat)
Casein	318.02	318.02
Gelatin	60	60
Corn starch	330.99	330.99
Microfine cellulose[a]	100	100
Kaolin	60	60
Fish oil	80	0.00
Pork lard	0.00	80
Mineral and vitamin suplement[b]	39.29	39.29
Betain	10	10
BHT[c]	0.20	0.20
Total	1000	1000

[a]Rhoster Indústria e Comércio Ltda.; [b]Guaranteed levels (kg/product): vit. A – 1,000,000 IU; vit. D3 – 312,500 IU; vit. E – 18,750 IU; vit. K3 – 1,250 mg; vit. B1 (thiamine) – 2,500 mg; vit. B2 (riboflavin) – 2,500 mg; vit. B6 (pyridoxine) – 1,875 mg; vit. B12 – 4 mg; vit. C – 31,250 mg; nicotinic acid – 12,500 mg; calcium pantothenate – 6,250 mg; biotin – 125 mg; folic acid – 750 mg; choline – 50,000 mg; inositol – 12,500 mg; iron sulphate – 6,250 mg; copper sulphate – 625 mg; zinc sulphate – 6,250 mg; manganese sulphate – 1,875 mg; sodium selenite – 13 mg; calcium iodate – 63 mg and cobalt sulphate – 13 mg; [c]Butylated hydroxytoluene.

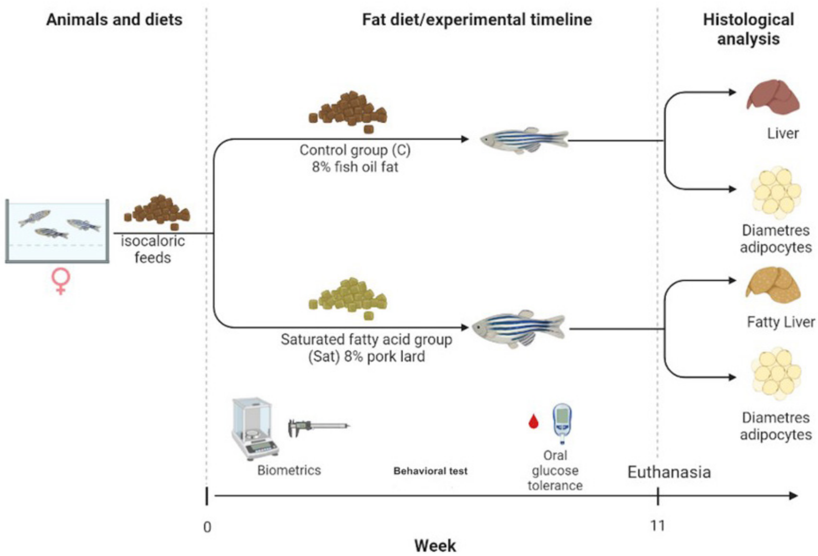

Figure 1. Timeline to evaluate the effect of imposing isocaloric diets with different lipid sources on zebrafish.

Table 2. New tank behavioral test assessing behavioral parameters of zebrafish under two diet protocols, Control Group (C) and Saturated fatty acid group (Sat) (Ethos Vision XT - video tracking NBT Ltd.).

Parameters	C	Sat	P
Total distance traveled (cm)	692.6 ±173.02	600.2 ± 158.4	0.775
Average speed (cm/s)	31.6 ± 10.7	20.7 ± 4.6	0.534
Time spent at depth (s)	335.6 ± 12.3	334.8 ± 8.5	0.603
Immobility time (s)	312.8 ± 12.7	304.01 ± 14.9	0.713

cm - centimeters; cm/s - centimeters/second; s – seconds; value of P≤0.05. Application of Student's t-test, n=20, P≤0.05.

index (BMI) was calculated as follows: BMI = body weight (grams) ÷ total body length^2 (centimeters).

In the last week of the experiment, a novel tank test was performed to evaluate the behavioral responses in fish. For this, 20 fish were removed from trial aquaria and transported to another under the same experimental conditions. The animal's behavior in an unfamiliar environment was recorded for 12 minutes, discarding the first and last 2 minutes to ensure adaptation and valid results (Table 2). The recording was analyzed by the EthoVisionXT software (Video Tracking - NBT Ltd.) which provided data related to the distance traveled (cm), average speed (cm/s), time spent at the bottom of the aquariums and immobility time (s) that corresponded a "freezing response", allowing for interpretations of the stress and anxiety levels (Table 2).

Fish underwent an oral glucose tolerance test after a 16-hour fasting time. The fish were first sedated by immersion in a 325 mg/l lidocaine solution prepared with aquarium water and glucose was measured using a portable glucometer (Accu-Chek Guide-Roche) (Zang et al., 2015). The first glucose measurement represented fasting glucose (T0). Following, a 1.25 mg/g glucose solution was administered orally with a micropipette (Zang et al., 2018) and glucose was measured after 30 min. (T30), 60 min. (T60) and 120 min. (T120). To reduce fish mortality during sampling, each fish was sampled twice, resulting in 8 measures from each time. To minimize the impact of the anesthetic solution on the glucose levels, all the fish were anesthetized, regardless of whether blood was collected.

2.3. Euthanasia

After collection of blood for glucose measurement, all fish were euthanized with an overdose of anesthetic followed by immersion in cold water for at least 10 minutes after cessation of all opercular movement was observed.

2.4. Histological analyses

For histological evaluation, the fishes after euthanasia were immersed in an aqueous formaldehyde 10% buffered solution, for at least two weeks. After fixation, the material was processed, and longitudinal and transversal slices were stained with hematoxylin and eosin. From these slices, photomicrographs of the liver and adipose tissue were obtained. Through stereological technique, hepatic

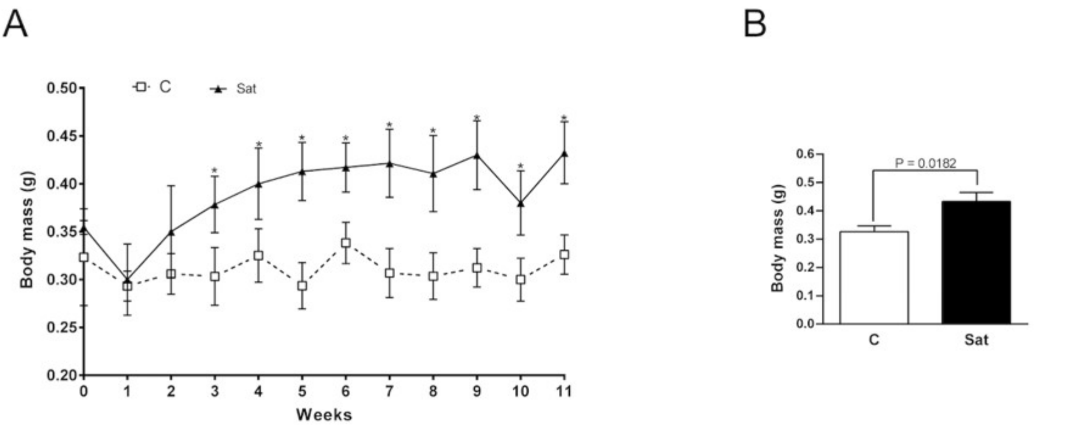

Figure 2. Body mass (mean ± SE) of zebrafish from the C and Sat groups over the 11 weeks of the experiment (A) and at the end of the last week (B). C = Control group, Sat = Saturated fatty acid group; Application of Student's t-test, n = 20, between groups P=0.0182, P≤0.05. Asterisks over the weeks, between the two groups, represent statistical difference, P≤0.05.

steatosis was quantified through the density of volume (Vv of hepatic steatosis), when a test system was used with 36 points (Hally, 1964) being: Vv [steatosis, liver] = Pp [steatosis, liver] / PT; being, Pp the number of points that touched the fat droplets and PT the total number of points in the test system (Catta-Preta et al., 2011) (Equation 1).

$$Vv = \frac{Pp}{Pt} \quad (1)$$

Micrographs of adipose tissue were used to conduct a morphometric analysis of the adipocytes. The mean diameters of adipocytes were measured from the digital images of the visceral and subcutaneous adipose tissue using the ImageJ software (version 1.53t, August, 2022, USA).

2.5. Statistical analysis

The data were expressed as means and standard error (SE). The data were tested for normality and homogeneity of variances (Kolmogorov-Smirnov). The experimental groups were compared using the unparied Student's t-test. All analyses were conducted using GraphPad Prism software (Prism 6.0c for Mac, GraphPad Software, La Jolla, CA). Differences were considered significant with P≤0.05.

3. Results

The analyses showed that the Sat group animals presented an increase in body mass with a difference compared to the C group (P≤0.05), from the third week of the diet, and this difference persisted until the end of the experiment (Figure 2A). At the end of the last week, the Sat group (0.43 ± 0.15g) presented a body mass 32% higher (P=0.0182) than the body mass of the control group (Figure 2B). On the other hand, the body mass index (BMI) did not show a significant difference between the groups (Figure 3).

The C and Sat groups did not show significant differences in oral glucose tolerance tests (TOTG) at T0, T30, and T120.

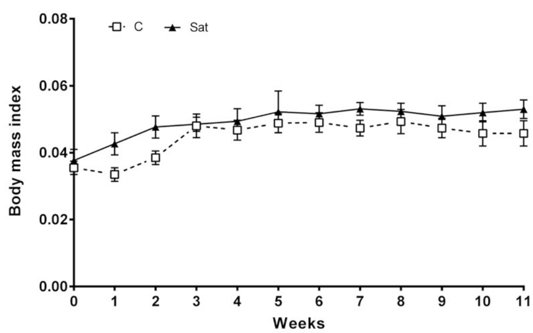

Figure 3. Body mass index (BMI) of zebrafish over the 11 weeks of the experiment. C = Control group, Sat = Saturated fatty acid group; Application of Student's t-test, n = 20, P≤0.05. The consumption of a diet rich in saturated fatty acids did not generate signs related to stress and anxiety in the zebrafish (Table 2).

However, TOTG demonstrated an increase in blood glucose at T60 (A) in the glycemic curves of the Sat and C groups, with statistical significance (Figure 4). In graph B, the area under (AUC) the TOTG curve showed no statistically significant difference in glucose levels between the Sat and C groups at the end (Figure 4).

From the stereological analysis of the liver, we observed an increase (P=0.0086) in hepatic steatosis in the Sat group compared to the control group (Figure 5).

Despite the Sat group animals displaying a greater deposition of adipose tissue, both visceral and subcutaneous, the average diameter of the adipocytes was similar between the two groups (P≤0.05).

4. Discussion

The results of this study showed that consumption of a diet high in saturated fatty acids can lead to an increase in body mass and hepatic steatosis. However, no apparent

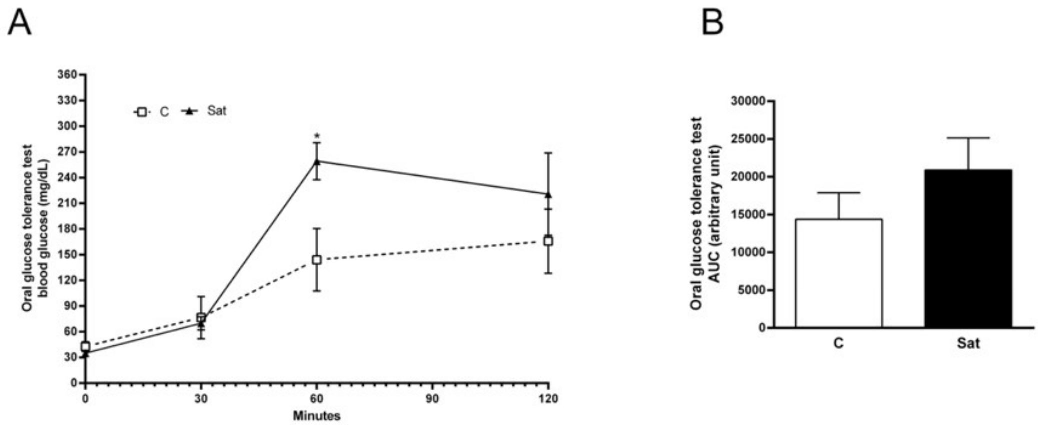

Figure 4. Oral Glucose Tolerance Test (TOTG) of the last week of the feeding protocol in zebrafish. Glucose curve with T0 (immediately before the oral administration of glucose) and 30 (T30), 60 (T60) and 120 (T120) minutes after glucose administration (A). Graph of the area under the curve (B). Application of Student's t-test, n = 20, P≤0.05. Asterisks along the times, between the two groups, represent statistical difference, P≤0.05.

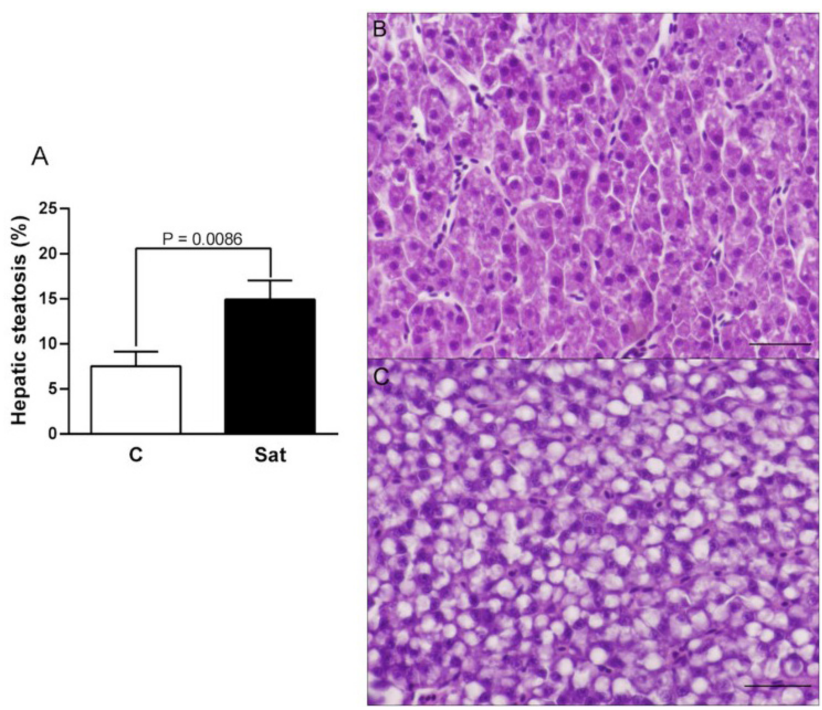

Figure 5. Quantification of hepatic steatosis (%) of the animals from group C and Sat, at the end of 11 weeks of the experiment (A). Representative photomicrograph of the liver of an animal from the Control group (A) and from the Sat group (B). Hematoxylin and Eosin, Bar = 30 micrometers. Application of the Student's t Test, n = 20, with P = 0.0086 between the groups, P ≤ 0.05.

effects were observed on the behavior and glucose metabolism of the animals involved in this study. This is an important scientific finding, as it shows that saturated fat consumption should not be seen as the sole factor responsible for metabolic and morphological changes in zebrafish. Therefore, further research is needed to better understand the role of saturated fatty acids.

Despite technological advances allowing us to conduct nutritional research involving zebrafish, these are still far from ideal. Thus, it is necessary to develop methods that allow for greater control over the nutrients ingested by zebrafish, ensuring both quality and the exact amount of each component, thus generating better results (Fowler et al., 2019). Most research using zebrafish has used practical diets, with pure or supplemented commercial feeds, which have had limited balance, as well as inconsistent composition, preventing control of the nutrients and their nutritional effects (Fowler et al., 2019). In mammals and zebrafish, specific nutrients and dietary ingredients, or the lack thereof, potentially altered physiology, behavior and molecular pathways (Gómez-Requeni et al., 2010; Meguro and Hasumura 2018). Therefore, the use of isocaloric diets of different compositions was one of the relevant factors for the execution of this work, as it ensured the improvement of nutritional and metabolic performance. Reducing or eliminating nutritional problems such as deficiencies, imbalance, or inadequate assimilation of nutrients. We consider that these diets could cause metabolic and nutritional disturbances, thus facilitating the understanding of their pathogenesis. As well as they would influence chronic inflammatory diseases and metabolic homeostasis. Finally, determinants for understanding how nutrition would affect the cell structure and function of specific organs in zebrafish.

The zebrafish has been a powerful model for diet-induced obesity, offering multiple advantages over others (Hölttä-Vuori et al., 2010; Oka et al., 2010). Similar to humans, zebrafish had an increase in weight gain, adiposity, and serum triglycerides when fed a high-fat diet, as well as showed precociousness for the occurrence of metabolic diseases (Hölttä-Vuori et al., 2010; Oka et al., 2010).

In this context, from our results, and as discussed by (Hölttä-Vuori et al., 2010; Oka et al., 2010) in their research, focusing on the study of metabolic diseases, it was possible to observe how much the diet based on semi-purified and purified diets was important. Based on our results in metabolic diseases (Mendes et al., 2023), it was observed that semipurified and purified diets were relevant (Hölttä-Vuori et al., 2010; Oka et al., 2010). This is because they accurately assessed the effects of manipulating macro and micronutrients (Fowler et al., 2019).

In relation to palmitic acid, female zebrafish, consuming a commercial diet supplemented with gluten and pork lard as a source of saturated fatty acids, had an increase in body mass relative to animals receiving commercial feed, supplemented with gluten and fish oil (Meguro and Hasumura, 2018). In the present work, we opted to complement such results by formulating semi-purified diets, to study the specific effects of replacing the lipid source, through isoenergetic diets. The main advantage of using isoenergetic diets was to know that the effects arising from the type of lipid provided, and not the amount of energy ingested or a possible imbalance of other macro or micronutrients, did not interfere with the results.

With regard to gender, we highlighted that zebrafish, both males and females, exhibited different behaviors, pharmacological and nutritional responses (Meguro and Hasumura, 2018). Therefore, we chose to use only females in this experiment. This is because in females, the decreased susceptibility to weight gain was attributed to the protective effects of estrogens, acting against the harmful effects of diet-induced obesity, further increasing energy expenditure in response to a higher amount of dietary fat (Clevenger et al., 2014; Palmer and Clegg, 2015). In the zebrafish, genes associated with metabolism and oxidative stress showed a gender-dependent response to carbohydrates in the diet (Robison et al., 2008). Therefore, one may assume that in the zebrafish, energy expenditure and the metabolism associated with the intake of fatty acids in the diet was also gender-related (Fowler et al., 2021; Fuente-Martín et al., 2013; Stubbins et al., 2012).

The diet-induced obesity zebrafish model utilized the same pathways as mammals (Meguro et al., 2019). Additionally, we observed that in these fish, consumption of saturated fatty acids had greater potential to trigger metabolic syndrome, when compared to consumption of polyunsaturated fatty acids. This information is pertinent and useful in providing nutritional guidance that reduces the risk of metabolic syndrome in humans (McCracken et al., 2018).

It was expected that diets rich in saturated fat would promote an increase in the body mass index (BMI) of the zebrafish compared to a control diet (Carnovali et al., 2018). However, our study showed that the groups maintained similar BMI, suggesting that the diet would not significantly influence the change in the BMI of the fish in question. Therefore, further studies would be necessary to determine the relationship of this diet to other biometric factors. Results like this have already been described in previous work when evaluating the Body fat volume ratio (Meguro et al., 2019). Thus, we understand and emphasize that in fish, body growth in adult individuals occurred differently than observed in mammals.

In zebrafish with hyperglycemia induced by continuous exposure to glucose, behavioral changes such as anxiety and memory impairments occurred, and these changes were reversed after the medical resolution of hyperglycemia (Santos et al., 2018). In the overfeeding commercial feed model, there was an increase in weight, hepatic steatosis and hyperglycemia, accompanied by neuroinflammation (Ghaddar et al., 2020). In the behavioral context, there was no difference in the distance traveled, but the overfeeding group showed an increase in inactive state (Ghaddar et al., 2020) . We emphasize that the behavioral changes in zebrafish exposed to continuous glucose exposure could promote a reduction in overall activity, less stress tolerance and less swimming activity. Above all, no behavioral changes were noted in the present study.

The short-term feeding of a supplemented diet with egg yolk also caused memory impairment and anxiety in zebrafish, without changes in locomotor parameters and vertical exploratory capacity (Picolo et al., 2021), as well as in our study. Similarly, (Meguro et al., 2019) found impaired cognitive function in animals that consumed high-fat ration, however, as in fish, consumption of this diet did not alter the distance traveled and average velocity. Similarly, cognitive functions were impaired in animals that consumed a high-fat diet (Meguro et al., 2019). In fish, the consumption of this diet did not alter the distance traveled or the average speed, indicating that cognitive functions were not compromised in zebrafish. There was an alteration in the results of the tests, indicating cognitive disturbances due to the consumption of a hyperlipid diet, and also an alteration in the expression of 97 telencephalon genes, some of them involved in the maintenance of the blood-brain barrier and oxidative stress (Meguro et al., 2019). Despite not having evaluated telencephalon function, the results obtained in our study, together with the available information, suggest that the consumption of hyperlipid diets promoted behavioral changes, consistent with telencephalon impairment. Up to this point, it has not been possible to extrapolate to zebrafish whether the responsible for the cognitive decline was directly due to the consumption of a hyperlipid diet, independently of the type of lipid, or whether the responsible was the consumption of some specific fatty acid, such as saturated fatty acids, already described in mammals (Santos et al., 2018). It has also not been possible to discard the effects of hyperglycemia on these behavioral changes (Santos et al., 2018). The large variation of behavioral results was related to the chosen model; therefore, it would be of extreme importance that new studies were carried out.

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The results indicated that the consumption of high-fat diets promoted behavioral changes in zebrafish. However, it was not possible to determine whether the cognitive decline in zebrafish was specifically caused by the consumption of a high-fat diet or by saturated fatty acids, which have been described as harmful in mammals (Santos et al., 2018). These diets, in addition to causing cognitive impairment, altered the gene expression related to beta-amyloid precursor protein, precursors of brain damage, and cerebrovascular pathologies (Constantinescu et al., 2011; Taha et al., 2012; Nakajima et al., 2020; Bernal-Vega et al., 2023; Raheem et al., 2023). Lipid-rich diets trigger brain responses characterized by changes in phospholipid and fatty acid concentrations, pro-apoptotic inflammatory mediators, and synaptic loss, contributing to cognitive impairment (Nakajima et al., 2020). These diets cause morphological changes due to the presence of atherosclerotic plaques in cerebral vessels and amyloid deposition, which impair normal brain function and highlight potential therapeutic targets for neurodegenerative diseases (Bernal-Vega et al., 2023).

Comparing an enriched diet with plamitic acid to a commercial diet with the same amount of fatty acids (Park et al., 2019) found that palmic acid was essential for the development of hepatic injury, despite both diets leading to increased body mass. In accordance with Palmer and Clegg (2015), we observed that palmic acid intake caused hepatic steatosis, highlighting especially that it was not an excessive intake. Our diets were isocaloric, both presented 8% of fat and still promoted weight gain accompanied by hepatic steatosis.

Different from observed in other models (Park et al., 2019; Zang et al., 2015), we observed through the TOTG that 11 weeks of feeding with 8% pork lard did not cause hyperglycemia and glucose intolerance (Zang et al., 2018). This result raised a discussion about this topic, as the absence of glucose intolerance could indicate that in the adult zebrafish, normal fat consumption occurred even when the diet was rich in saturated fatty acid. Another sensitive point was the execution of the TOTG in the zebrafish, as we assessed the glycemia at different moments in the same animal, and the size of the fish and the available blood volume limited the number of seriated collections. Despite puncturing only 0.6 to 1 ul, the minimum volume necessary for the glucometer reading, we cannot discard the interference of some compensatory response.

5. Conclusion

The study conducted with zebrafish was an experimental model that was able to establish the relationship between diet and increased body mass. The results showed that despite diets having the same fat content, diets rich in unsaturated fatty acids (Sat) presented greater obesogenic potential. This increase in body mass was accompanied by hepatic steatosis. In addition to the changes in the (Sat) group, it was possible to ascertain that the dietary protocol did not cause stress and did not alter glucose metabolism. These results ensure the understanding and treatment of metabolic alterations related to diet, as well as for the prevention of these problems.

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