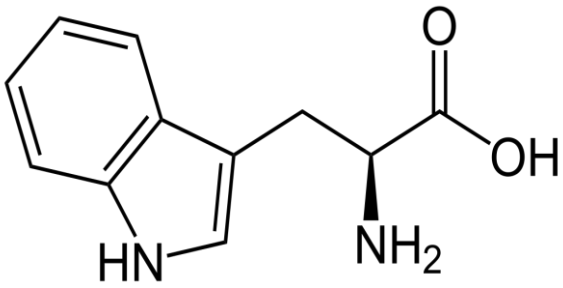

Evaluating effects of dietary tryptophan on growth performance, feed utilization, biochemical parameters, gene expression and behavior of zebrafish juveniles

Cláudia Alexandra da Mota Teixeira
Mestrado em Recursos Biológicos Aquáticos
Departamento de Biologia
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Orientador
Professor António Paulo Alves Ferreira de Carvalho
Professor Auxiliar da Faculdade de Ciências da Universidade do Porto
Investigador no Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR)

Coorientador
Professora Maria Helena Tabuaço Rego Martins Peres
Investigadora no Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR)

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FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto,_____/_____/_____

“Find what you love and let it kill you.
Let it drain you of your all. Let it cling onto your back and weigh you down into eventual nothingness.
Let it kill you and let it devour your remains.
For all things will kill you, both slowly and fastly….”

- Charles Bukowski

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Para os que viram o início mas não puderam estar presentes no fim… à minha Bisavó e ao meu Avô

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Resumo

O Triptofano (Trp) é um amino ácido essencial utilizado para a síntese proteica, e várias funções metabólicas. Ademais, é o precursor da síntese da serotonina, um neurotransmissor com papel relevante em várias respostas comportamentais e fisiológicas. A suplementação de Trp em dietas para produção de peixes tem sido uma estratégia utilizada para permitir aumentar a atividade serotonérgica contribuindo para a mitigação do stress associado às práticas de aquacultura.

Assim sendo, este trabalho teve como objetivo avaliar os efeitos de cinco dietas com diferentes níveis de Trp em juvenis de peixe zebra, ao nível do crescimento e da utilização do alimento, de parâmetros bioquímicos, bem como da expressão de alguns genes selecionados e do comportamento locomotor.

Para este propósito, realizou-se um ensaio de crescimento com juvenis de peixe-zebra, utilizando cinco dietas isoproteicas (40%DM) e isolipídicas (8%DM) suplementadas com diferentes níveis de Trp desde 0.00% a 3.00%DM, para um total de Trp de 0.19, 0.38, 0.76, 1.52 e 3.04%DM (dietas Trp0o, Trp2, Trp4, Trp8, Trp16, respetivamente). Vinte grupos homogéneos de 20 peixes foram formados (com um peso inicial médio de 65.9 mg). As dietas foram aleatoriamente distribuídas e testadas em quadruplicado, com os peixes a serem alimentados duas vezes por dia, seis dias por semana, durante seis semanas. No final do ensaio, seis peixes de cada réplica dos grupos alimentados com as dietas Trp0, Trp4 e Trp16 foram amostrados, tendo sido recolhidos cérebro, músculo e fígado, para a análise do nível de serotonina (cérebro), da atividade da acetilcolinesterase (cérebro e músculo), da expressão de genes selecionados (fígado). Os genes selecionados estão relacionados com as vias de acreção e degradação proteica (IGF1, TOR, UBE2H), a via da serotonina (recetores da serotonina: Htr2cl1, Htr1aa; transportador de serotonina: Slc6a4a; hidroxilase do Trp: TPH1a, TPH1b, TPH2a, TPH2b) e a regulação das respostas protetoras celulares contra stress eletrofilico e oxidativo (Keap1, Nrf2). Quarenta e oito peixes de cada um dos referidos tratamentos foram usados para análise do comportamento locomotor através de um sistema de vídeo rastreio com um software apropriado. Finalmente, os peixes restantes foram amostrados para análise da composição corporal.

Os resultados indicam que os níveis de triptofano testados não influenciaram o crescimento, a ingestão do alimento, nem a eficiência alimentar. Contudo, as Retenções azotada e energética apresentaram uma resposta quadrática aos níveis de Trp da dieta.

Uma resposta quadrática aos níveis de Trp da dieta foi igualmente observada, relativamente à atividade da acetilcolinesterase no cérebro, mas não no músculo. Além disso, no cérebro registou-se um aumento linear dos níveis de serotonina com o aumento do Trp na dieta.

As dietas afetaram também a expressão génica; Trp4 diminuiu expressão do gene Htr2cl1, e Trp4 e Trp16 diminuiram a expressão do gene Htr1aa. Encontraram-se ainda respostas lineares positivas para os genes Keap1 e TOR, e uma resposta linear negativa no TPH1a em função do nível de Trp da dieta.

Detetaram-se igualmente diferenças no comportamento locomotor dependentes da dieta, com os peixes alimentados com a dieta Trp4 mostrando um comportamento médio que poderá ser associado a um menor stress e os peixes alimentados com as dietas Trp0 e Trp16 exibindo um comportamento médio que poderá ser associado a maior stress.

Palavras-Chave *Danio rerio*; Acetilcolinesterase; Serotonina; Nutrição; Parâmetros Zootécnicos; Amino ácidos; Expressão génica; Comportamento.

Abstract

Tryptophan (Trp) is an essential amino acid, used for protein synthesis and several metabolic functions. Moreover, it is the only precursor of serotonin synthesis, a neurotransmitter with relevant roles in physiological and behavioral responses. Increasing levels of dietary Trp have been shown to increase brain serotonin levels through a dose-response mechanism. Supplementation of Trp in fish diets is being explored as a strategy to mitigate stress associated with rearing conditions in aquaculture practices.

Thus, this study aimed to evaluate the effects of increasing levels of dietary Trp in zebrafish juveniles by assessing growth performance and feed utilization, biochemical parameters, as well as gene expression and locomotor behavior.

For this purpose, a growth trial was conducted with zebrafish juveniles fed isoproteic (40%DM) and isolipidic (8%DM) diets supplemented with five graded levels of Trp ranging from 0.00 to 3.00% DM, for a total of 0.19, 0.38, 0.76, 1.52, 3.04%DM (diets, Trp0, Trp2, Trp4, Trp8, Trp16, respectively). Twenty homogenous groups of 20 fish (initial body weight of 65.9 mg) were randomly distributed by each tank. Diets were tested in quadruplicate, with fish being fed twice a day, six days a week for six weeks. At the end of the trial, six fish from each replicate of groups fed Trp0, Trp4 and Trp16 were sampled and brain, muscle, and liver for serotonin analysis (brain), acetylcholinesterase activity (brain and muscle) and gene expression (liver). Gene expression measured in the liver followed the protein accretion and degradation pathways (IGF1, TOR, UBE2H), the serotonin pathway (serotonin receptors: Htr2cl1, Htr1aa; serotonin transporter: Slc6a4a; Trp hydroxylase: TPH1a, TPH1b, TPH2a, TPH2b) and the major regulator of cellular protective responses against elecrophilic stress and oxidative stress (Keap1, Nrf2). Forty-eight fish from each of the referred dietary treatments were analyzed in a video tracking system and locomotor behavior was assessed. All remaining fish were sampled for whole-body composition analysis.

Results indicate that tested dietary levels of Trp did not influence growth performance nor feed utilization, though quadratic tendencies were encountered for nitrogen retention and energy retention. Increasing levels of Trp linearly increased brain serotonin levels. A quadratic response was found for brain acetylcholinesterase activity in relation to Trp levels, but muscle acetylcholinesterase was not affected.

Dietary treatment also influenced gene expression, Trp4 lowered relative expression of Htr2cl1, and Trp4 and Trp16 lowered relative expression of Htr1aa; moreover increasing linear

tendencies with the Trp dietary level were encountered in TOR and Keap1 relative expression, and a negative linear expression was encountered for TPH1a.

Also, distinctive locomotor behavior were detected depending on dietary treatment with fish fed Trp4, which had an average behavior which may be associated to a less stressed behavior, while Trp0 and Trp16 were associated with a more stressed behavior.

Key Words: *Danio rerio*; Acetylcholinesterase; Serotonin; Nutrition; Zootechnical Parameters; Amino Acids; Gene Expression, Behavior

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List of Abbreviations

2D Two-Dimensional

5-HT Serotonin

5-HIAA 5-Hydroxyindoleacetic acid

AAA Aryl Acylamidase Activity

AAAD Aromatic Amino Acid Decarboxylase

AANAT Aryl-Akylamine-N-Acetyltransferase

ABW Average Body Weight

ACh Acetylcholine

AChE Acetylcholinesterase

Ag Silver

AgCl Silver Chloride

ANOVA Analysis of Variance

BBB Blood- Brain-Barrier

BChE Butyrylcholinesterase

BEWS Biological Early Warning System

cDNA complementary Deoxyribonucleic Acid

ChE Cholinesterases

CRF Corticotropic Release Factor

DGI Daily growth index

DHBA 3,4-hydroxybenzylamine

DM Dry Matter

DNTB 5-5'-dithio-bis-[2-nitrobenzoic] acid, Ellman's Reagent

E Energy

E1 Ubiquitin-activating enzymes

E2 Ubiquitin-conjugating enzymes

E3 Ubiquitin-ligases

EAA Essential Amino Acid

EI Energy Intake

ER Energy Retention

F Forward

FBW Final body weight

FE Feed Efficiency

FI feed intake.

FM Fresh Matter

GH/IGF Growth Hormone/ Insulin Growth like-factor

HIOMT Hydroxyindole-O-methyltransferase

HPLC High Performance Liquid Chromatography

HPLC-ECD High Performance Liquid Chromatography with electrochemical detection

Htc2cl1 Serotonin receptor Htc2cl1

Htr1aa Serotonin receptor Htr1aa

IBW Initial Body weight

IDO Indoleamine 2,3-dioxygenase

IGF Insulin growth like-factor

IGF1 Insulin growth-like factor 1

Keap1 Kelch-like ECH-associated protein 1

LNAA Large Neutral Amino Acids

MAO Mono-amine Oxidase

N Nitrogen

NaOH Sodium Hydroxide

NaCl Sodium Chloride

NCBI National Centre for Biotechnology Information

NI Nitrogen Intake

NR Nitrogen Retention

Nrf2 Nuclear factor (erythroid-derived 2)-like 2

PCR Polymerase Chain Reaction

PER Protein Efficiency ratio

PrChE Propionylcholinesterase

qPCR Reverse Transcriptase Quantitative Real Time Polymerase Chain Reaction

R Reverse

RAS Recirculating Aquaculture System

ROS Reactive Oxygen Species

RNA Ribonucleic acid

SEM Standard Error of the Mean

Slc6a4a Serotonin Transporter Slc6a4a

SSRI Selective Serotonin Reuptake Inhibitor

TDO Tryptophan 2,3-dioxygenase

TNB 5-Thio-2-nitrobenzoate

TOR target of rapamycin

TPH Tryptophan Hydroxylase

TPH1a Tryptophan hydroxylase 1 a

TPH1b Tryptophan hydroxylase 1 b

TPH2a Tryptophan hydroxylase 2 (variant 1)

TPH2b Tryptophan hydroxylase 2 (variant 2)

Trp Tryptophan

UBE2H Ubiquitin-conjugating enzyme E2H

UPP Ubiquitin Proteosome Pathway

WG Weight gain

Chapter One

General Introduction

Zebrafish (*Danio rerio*, Hamilton 1822)

Zebrafish is a small tropical freshwater teleost (Nasiadka and Clark, 2012), belonging to the Cyprinidae family, it is native from Southeast Asia countries (Bangladesh, Myanmar, India, Nepal, and Pakistan). It presents a fusiform and laterally flattened body with a terminal oblique mouth directed upwards and the lower jaw is more protruded than the upper. Its name is due to the five alternating blue-black and silvery-yellow longitudinal stripes throughout its extension (Spence *et al.*, 2008).

This species inhabits slow-moving waters and can be found in rice-fields (Talwar and Jhingran., 1991) and lower reaches of streams (McClure et al., 2006), usually waters with high transparency, It has wide temperature plasticity tolerating temperatures ranging from 6ºC during the winter to 38ºC during the summer, in their natural habitat. Zebrafish are omnivorous and euryphagous as they eat a great variety of items from zooplankton to insect eggs, according to observations made in their natural habitat as well as gut content analysis, moreover, adults may prey on eggs and larva from their own species (Dutta, 1993; McClure et al., 2006; Lawrence, 2007; Spence *et al.*, 2007, 2008; Watts *et al.*, 2012).

Zebrafish has been used as an ornamental fish, however in recent years it has emerged as one of the major research models in a wide range of areas such as biomedicine, toxicology, vertebrate biology and physiology, pharmacology, genetics, neuroscience and behavioral studies (Vascotto *et al.*, 1997; Grunwald and Eisen, 2002; Rubinstein, 2003; Amsterdam and Hopkins, 2006; Lawrence, 2007; Spence et al, 2008; Bakkers, 2011; Kinth *et al*., 2013). Its utilization as a biological model has been growing since 1951 after the first paper using it was published. However, the use of zebrafish in scientific research has exponentially grown since 2000, resulting in around 3000 indexed in PubMed until 2015 (Figure 1) (Kinth et al., 2013; Geisler *et al.*, 2016).

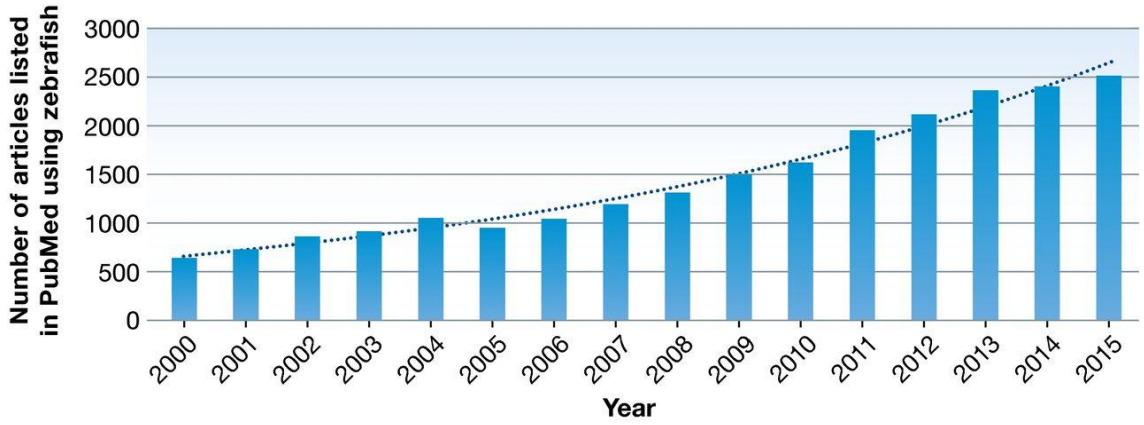

Figure 1 Number of scientific articles in PubMed using zebrafish from 2000-2015 (Geisler et al., 2016)

Zebrafish has a distinct set of characteristics that allow it to be an excellent research model. It has a small size, not exceeding 5 cm length in its 2 to 4 years life span (Schilling, 2002). This allows keeping a large number of individuals in small facilities, making it easier to perform large scale experiments with a higher number of replicas. Moreover it is cheaper to rear and maintain than other vertebrates models (Lieschke and Currie, 2007; Spence *et al.*, 2008). Also, zebrafish can easily form a shoal, which is important to maintain social interactions and cohesiveness resulting in an undisturbed natural behavior (Amorim, 2014).

Zebrafish specimens have sexual dimorphism, with males having golden stripes and females having silver stripes and a more prominent abdomen (Figure 2). Zebrafish are asynchronous spawners, meaning females are able of ovulating regularly (Spence et al. 2008) and therefore they can spawn almost every day. In addition, this species has a short generation time (about 3 months), facilitating the possibility of transgenerational studies (Maack & Segner, 2003). Finally, embryos develops externally and are transparent, allowing the observation of the developmental stages and the direct visualization of markers (Kimmel *et al.*, 1995; Deo & MacRae, 2011).

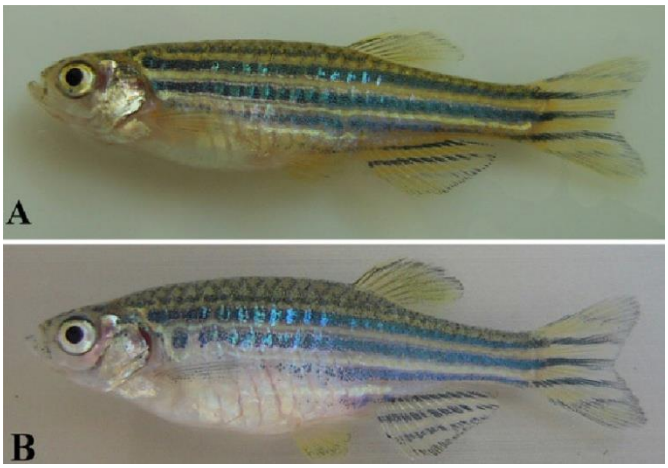

Figure 2 Male (A) and Female (B) Zebrafish (Avdesh *et al.,* 2012)

In the laboratory environment, zebrafish culture is usually performed in Recirculating Aquaculture System (RAS), which reduces water uses when compared to flow-through systems (Lawrence and Mason, 2012). Laboratory-reared zebrafish juveniles and adults are fed commercial dry feeds, most commonly food flakes used for ornamental fish, sometimes supplemented with *Artemia naupilli*. Food availability and feeding frequency can affect both growth and reproductive performances (Lawrence *et al.,* 2012). Feeding practices tend to vary between facilities, meaning that diet is an uncontrollable variable in most studies. The importance of controlling external variables such as diet can influence results and experimental outcomes (Tye, 2015, Fowler *et al.,* 2019).

Studies on zebrafish nutrition are scarce and the nutritional requirements of zebrafish are largely unknown. Basic nutritional requirements such as essential amino acids (EAA) and essential fatty acids have yet to be determined. Therefore, a commonly accepted approach is that known requirements for other cyprinids can be used for zebrafish (Lawrence, 2007; Watts *et al.*, 2012; Fernandes *et al.*, 2016). Recently, Fernandes *et al.* (2016) found the protein requirements for zebrafish juveniles were about 40% (DM), slightly higher in comparison with other cyprinids, 34% for Jian carp (Liu et al., 2009), 30% to Indian carp (Jena et al., 2012), 30-45% for *Cirrhinus mrigala* and 34-37% for common carp (FAO, 2019). Regarding amino acids, the requirements for zebrafish juveniles in lysine and arginine were established at 2.2% and 1.95% of the diet (Tye, 2015).

As aforementioned, zebrafish is a well-established experimental model; however, when it comes to aquaculture, this is completely true. Nonetheless, it could serve as a potential replacement for traditional nutrition studies (Ulloa et al., 2011, 2013, 2014; Oyarbide et al., 2012; Hedrera et al., 2013; Ribas and Piferrer, 2013; Aleström and Winther-Larsen, 2016). Indeed, due

to its versatility and requirement for a small experimental space, the utilization of zebrafish would allow the evaluation of a higher number of dietary treatments in less time, lowering the costs with aquaculture experiments (Gómez-Requeni *et al.*, 2010, Fuents-Applegren, 2014).

Tryptophan

Tryptophan (Trp) is not synthesized by the organism; meaning it has to be supplied through diet with proper amounts (NRC, 2011). Teleost requirements for this amino acid are generally determined by growth dose-response curves, from 0.3-1.1% of dietary protein for *Cyprinus carpio* (Dabrowski, 1981; Tang *et al.*, 2013); 0.95-1.20% for *Cirrhinus mrigala* (Benakappa and Varghese, 2003; Ahmed and Khan, 2005); 0.4-0.71% for *Oncorhynchus mykiss* (Walton *et al.*, 1984, 1986; Kim *et al.*, 1987); 0.90-1.13 for *Labeo rohita* (Murthy and Varghese, 1997; Fatma Abidi and Khan, 2010). However, some inconsistencies have been reported regarding the requirements of this amino acid, which may be related to the fact that Trp is used for other functions aside from protein synthesis (Wu, 2009). Indeed, Trp requirements increase under stress conditions (Tejpal et al, 2009) as concentrations of Trp in plasma decrease (Aragão *et al.*, 2008; Costas *et al.*, 2008).

Deficiency in Trp can retard growth, and can cause anatomical deformities such as, scoliosis, a lateral curvature of the spine; lordosis, a dorsal deformity in V shape, as well as cataracts, opercular shortage, fin erosion and interference in the mineral metabolism (revised by Hosseini et al., 2017).

Aside from its role in protein accretion, Trp is also involved in numerous metabolic functions such as regulation of appetite, glucose homeostasis and immune and inflammatory responses (Pérez-Sánchez and Le Bail, 1999; Le Floc'h and Seve, 2007; Matte *et al.*, 2011; Yao *et al.*, 2011; Machado *et al.*, 2015; Azeredo *et al.*, 2017). Trp modulates neurological and immunological functions through different metabolites such as serotonin (5-HT) and melatonin (Wu, 2010). Trp is the only precursor of 5-HT, throuugh hydroxylation of the indole ring followed by decarboxylation of Trp (Katzung *et al.*, 2012), generally occuring in the gastrointestinal tract and brain, which can then be converted into melatonin (Figure 3). In teleosts, regulatory roles of Trp are not well explained in opposition to mammals; moreover, inconsistencies amongst studies strength the need for further research to fully understand its effects.

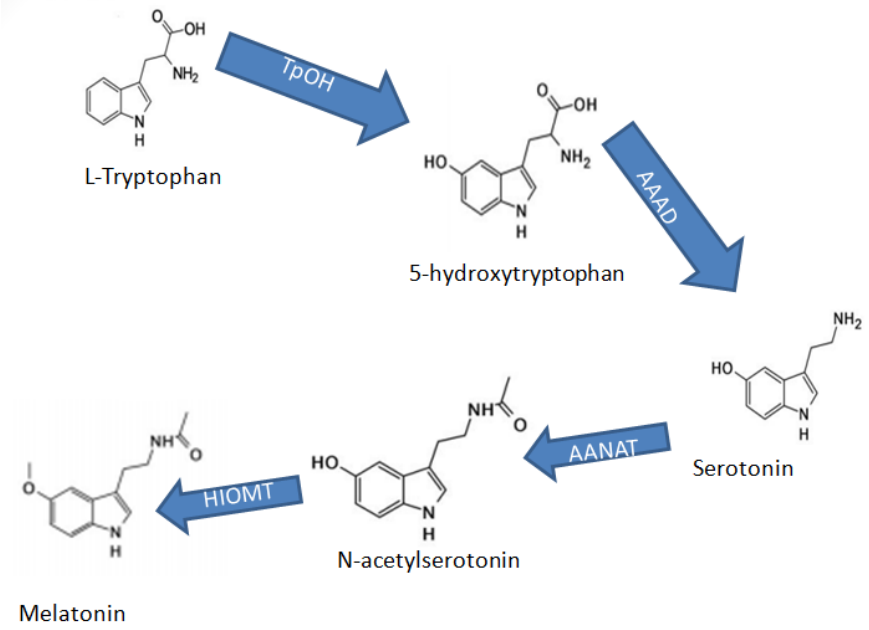

Figure 3Serotonin and Melatonin biosynthetic pathway from L-Tryptophan: TpOH or TPH- Tryptophan Hydroxylase, AAAD- Aromatic Amino Acid Decarboxylase, AANAT- Aryl-Akylamine-N-Acetyltransferase, HIOMT- Hydroxyindole-O-methyltransferase. Adapted from Hoseini et al., 2017

Catabolism of Trp generally occurs in the brain and liver through the kynurenine-niacin pathway which degrades 90% of the Trp that is not used for protein synthesis, and depends on deamination and decarboxylation to form kynurenine (Wu, 2013). The kynurenine-niacin pathway is not as well understood as the serotonin synthesis in teleosts, as fish aren't able to produce substantial amounts of niacin from Trp catabolism, which means this pathway should be mostly used to excrete excess Trp (Peters, 1991; Ng et al., 1997, Hoseini et al., 2017).

Trp is a large neutral amino acid (LNAA) and it is transported to the brain through the blood-brain-barrier (BBB) by a LNAA transporting protein (Pardridge and Oldendorf, 1977; Pardridge, 1983, 1988; Fernstrom and Wurtman, 1997; Johnston *et al.*, 1990, Aldegunde *et al.*, 1998, Höglund, *et al.*, 2007). Studies suggest that fish have a similar BBB that of mammals (Peyraud-Waitzenegger et al., 1979; Cserr and Bundgaard, 1984) and as Trp crosses the BBB it activates the serotonergic activity, increasing serotonin release, which can display endocrine and behavioral responses (Winberg *et al.*, 2001, Lepage *et al.*, 2002, 2003, Höglund *et al.*, 2005, 2007). Indeed, Trp is the limiting factor for serotonin synthesis (Johnston *et al.*, 1990; Herrero *et al.*, 2007), therefore disturbances in serotonin levels can be a result of Trp deficiency, which will result in affective disorders, anxiety, aggression, stress, eating disorders, among others (Johnston *et al.*, 1990; Herrero *et al.*, 2007; Le Floc'h *et al.*, 2011). Trp indirectly stimulates or inhibits the cortisol release (reviewed in Höglund *et al.*, 2017). Some studies indicate that supplementation of Trp in diets lead to a decrease in cortisol levels (Lepage *et al.*, 2002, 2003; Höglund, 2017).

However, Lepage *et al.* (2002, 2003) also showed that in undisturbed fish cortisol levels increased with Trp supplementation.

Several studies report that administrating Trp increases serotonergic activity, either by increasing 5-HT or 5-Hydroxyindoleacetic acid (5-HIAA), the main serotonin metabolite (Lepage et al., 2002, 2003; Hseu et al., 2003; Herrero et al., 2007; Höglund et al., 2007; Basic et al., 2013). Trp is also important for melatonin synthesis aside being the precursor for 5-HT. Trp harmonizes stress response, modulating behavior (Winberg and Lepage, 1998; Winberg *et al.*, 2001; Lepage *et al.*, 2002, 2005 a, b; Herrero *et al.*, 2007; Azpleta *et al.*, 2010; Hoseini *et al.*, 2017). Indeed, serotonin has been shown to have relevant roles in physiological and behavioral responses, such as stress reaction, social dominance, sexual behavior and reduction of aggressive behavior (Höglund *et al.*, 2005; Papoustsoglou *et al.*, 2005: Tejpal *et al.*, 2010; NRC, 2011; Hosseini et al., 2017). Previous studies have reported behavioral effects from Trp supplementation in diets such as decreased aggression, reduction stress response and attenuation of stress-induced anorexia (Winberg *et al.*, 2001; Hseu *et al.*, 2003; Höglund *et al.*, 2005, 2007; Wolkers *et al.*, 2012; Basic *et al.*,2013), However, previous studies on the effect of Trp on feeding behavior and/or growth showed contradictory results in superior vertebrates (Rosebrough, 1996; Haleem *et al.*, 1998; Seve, 1999 Hussein *et al.*, 2001; van Hierden *et al.*, 2004) and in fish (Winberg *et al.*, 2001; Hseu *et al.*, 2003; Papoustsoglou *et al.*, 2005).

Trp is also a limiting factor for melatonin synthesis, as the activity of the enzymes Aryl-Alkylamine-N-Acetyltransferase (AANAT) and Hydroxyindole-O-methyltransferase (HIOMT) depend on substrate concentration, and therefore Trp is crucial to assure enough substrate (Huether *et al.*, 1992). Melatonin has been indicated as conditioning growth performance, changing metabolism and blood parameters and regulating digestive capacity (Falcón *et al.*, 2010), however the most important roles of melatonin are the enhancement of humoral innate immunity status and the protection against the oxidative stress, thus improving welfare status in farmed animals (Lepage *et al.*, 2005b; López-Olmeda *et al.*, 2006; Cuesta *et al.*, 2008; Falcón *et al.*, 2010).

Excessive levels of Trp in diets have been reported to induce harmful effects such as lower growth performance (Murthy and Varghese, 1997; Ahmed and Khan, 2005; Ahmed, 2012), which can be interpreted as a possible toxic effect. However, further studies are needed to clarify it. Some authors attribute this negative effect of excessive levels of Trp, to an amino acid imbalance, as Trp and LNAA compete for the same transporter (Wu, 2013). Supplementation of Trp in diets affects the Trp/LNAA ratio, as Trp concentrations increase in the brain at the expense of a decrease in other LNAA (valine, leucine, isoleucine, tyrosine, and phenylalanine)

concentrations (Pardridge and Oldendorf, 1977; Johnston et al., 1990; Shen *et al.*, 2012; Martins *et al.*, 2013).

Locomotor Behavior

As aforementioned, Trp can affect locomotor behavior through its role in 5-HT synthesis. Indeed both Trp and 5-HT supplementation on teleosts resulted in similar effects (Hoseini et al., 2017). Alterations to standard behavior can have relevant implications, affecting not only animal survival but also its development and reproduction.

Behavior can be a tool for toxicity analysis and has gained relevance in ecotoxicological studies due to its higher sensibility (up until 1000 times higher) when compared to the conventional parameters such as survival or growth (Hellou, 2008). In fact, changes in standard behavior may occur with lower stimuli than those needed to elicit clear physiological effects (Faucher et al., 2008). Behavior is an individual effect of an organism and it is defined as the action or reaction to a set of conditions and circumstances (Hellou, 2011). Several behavioral patterns can be easily observed and quantified, such as locomotion, reproduction, feeding, aggressiveness, memory (Amorim, 2014, Blaser and Vira, 2014).

Monitoring fish behavior can present the opportunity to understand how the organism reacts to changes introduced in their surrounding environment. Thus, behavioral monitoring has some clear benefits, aside from the previously mentioned, having the possibility of recording of small videos, with a lower cost and a simplistic use, when compared to some physicochemical sensors, commonly used (Gerhardt, 1999; Park, et al., 2005).

Computer vision systems have been developed and, in the last decade, biological early warning systems (BEWS) based on locomotor behavior have grown due to the improvement of algorithms allowing the objective analysis of locomotion recordings. Some allow an automatic observation of behavioral patterns and can be carried out for single or multiple individual in 2D or 3D (Suzuki et al, 2003; Papadakis et al, 2012; Delcourt et al, 2013; Kulina et al, 2013; Bae and Park, 2014; Dell et al, 2014; Xia et al, 2018). These systems have used to interpret biological responses to chemicals as well as responses to external factors (ecological stressors) (Xia et al., 2018).

Zebrafish are very active and move almost continuously during the daylight period, which can be effective in video-tracking systems (Grillitsch *et al.*, 1999; Teles *et al.*, 2015). Moreover, zebrafish display a range of well-characterized social and defensive behaviors (behavior phenotyping) that can be very useful to characterize its behavior (Gerlai, 2003). Several

behavioral variables can be determined and analyzed, as stop frequency, time of movement, linear velocity, and angular velocity (Kane *et al.*, 2004; Blaser & Gerlai, 2006; Amorim, 2014). Additionally, other behavioral traits can be analyzed like memory, reflection, aggressiveness, cohesiveness when in a shoal, among others (Blaser & Vira, 2014). Zebrafish behavior has been mainly used to detect disturbances in the aquatic environment and in the screening of toxicants and drugs (Speiser *et al.*, 1996; Berghmans *et al.*, 2007; Gerlai *et al.*, 2006, 2008; Teles *et al*, 2015).

Cholinesterases

Cholinesterases (ChE) are enzymes that hydrolyze choline esters and are considered as one of the most efficient enzymes in nature (Sepčić et al., 2019). There are two types of ChE: acetylcholinesterase (AChE) and pseudocholinesterase such as butyrylcholinesterase (BChE) and propionylcholinesterase (PrChE) (Rodrigues et al., 2013a). ChEs exhibit an aryl acylamidase activity (AAA), which is inhibited by ChEs inhibitors. Furthermore, AAA is sensitive to other neurotransmitters such as serotonin and it is selectively inhibited by 5-HT (Balasubramanian and Bhanumathy, 1993; Toledo-Ibarra et al., 2013).

AChE is an enzyme responsible for the degradation of the neurotransmitter acetylcholine (ACh) into choline and acetic acid (Čolovic et al., 2013) (Figure 4). ACh is released into the synaptic cleft binds to receptors in the ionic channels of the postsynaptic cell membrane, which causes the opening of those channels and leads to a change of the postsynaptic cell membrane potential. AChE catalyzes the degradation of ACh, promoting its detachment from the postsynaptic cell receptors (Mesquita et al., 2011) and allowing the postsynaptic cell to return to a resting state (Čolovic et al., 2013). When AChE activity is inhibited, postsynaptic cells are overstimulated, which may lead to the death of the organism. Indeed, some toxic substances can inhibit AChE such as organophosphates and carbamates that can be present as environmental contaminants and natural toxins (Rodrigues *et al.*, 2013b). Moreover, the inhibition of ChE enzymes is related to neuromotor control and may affect locomotor activity in several species (Paul et al., 1998; Dam et al., 2000; Mesquita et al., 2011; Pereira et al., 2013).

ACh has a higher affinity for AChE than for pseudocholinesterases. Pseudocholinesterases have a higher affinity with other substrates, as for example butyrylcholine has higher affinity for BChE and propionylcholine for PrChE (Rodrigues et al., 2013a). Pseudocholinesterases can degrade ACh as well as other esters, however function of BChE is not known, though it's suggested to play a role in growth as well as helping in synaptic transmission

(Pezzementi et al., 2011). Zebrafish does not possess the gene for BChE (Bertrand et al, 2000; Richetti et al., 2011; Koening et al., 2016), which means degradation of ACh is performed only by AChE, indeed the AChE gene has already been detected and cloned in the brain of this teleost (Bertrand et al., 2001).

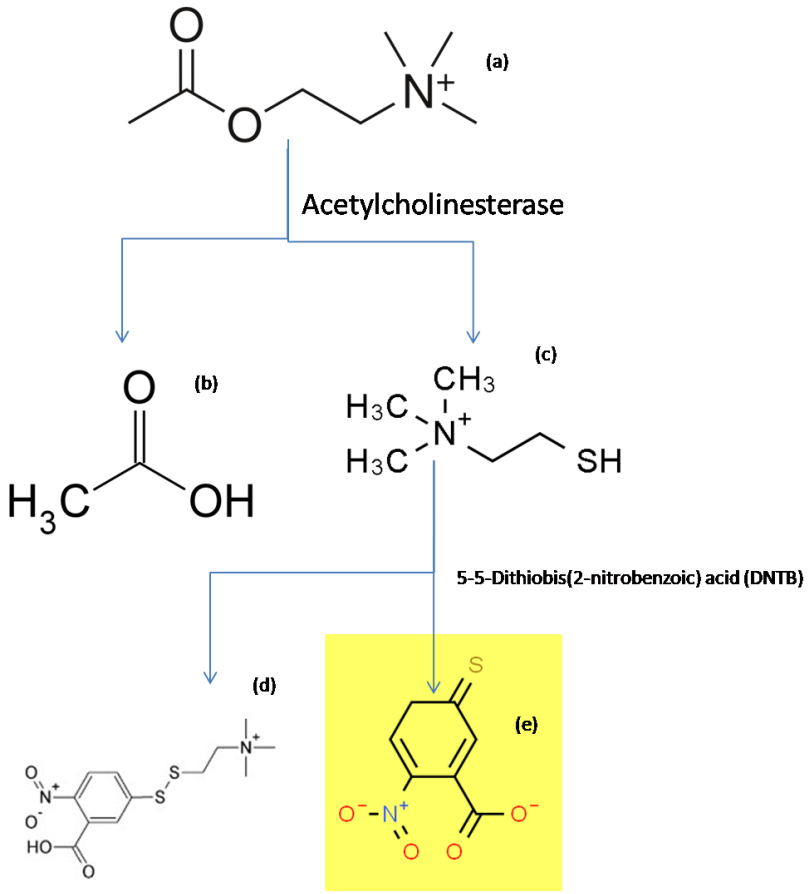

Figure 4 Acetylcholinesterase (AChE) hydrolyzes Acetylcholine (a) and forms Acetic Acid (b) and Thiocholine (c), which reacts with 5-5-Dithiobis(2-nitrobenzoic) acid (DNTB) releasing 2-Nitrobenzoate-5-mercaptothicholine (d) and 5-thio-2-nitrobenzoate (TNB) (e). TNB has a yellow color. Adapted from Badawy and El-Aswad, 2014.

As aforementioned, ChEs activity can be conditioned by other neurotransmitters such as serotonin. Indeed, studies reported that serotonin can inhibit AChE activity in rats (Oderfeld-Nowak et al., 1980; Gillet et al, 1985; Maura and Raiteri, 1986; Jackson et al., 1988; Nurrish et al., 1999). However, the relationship between the serotonergic and cholinergic systems is not well studied, affecting the knowledge of possible interactive effects of these two systems.

Gene Expression

Amino acids and their supplementation in diets can influence growth performance, in recent years interest in understanding the role of amino acids in growth, amino acid metabolism and protein turnover through evaluation of the expression of genes has increased (Panserat and Kaushik, 2010; Rolland et al., 2015). As aforementioned, Trp is not only used for protein synthesis but also 5-HT synthesis and other metabolic functions. Also, it has been suggested that Trp can have some antioxidant properties and some of its metabolites can act as reactive oxygen species (ROS) scavengers and modulating antioxidant enzymes. However, some contradictory data has been found, as excessive Trp levels can induce oxidative stress (reviewed by Hoseini et al., 2017).

The genes of interest for the present study were selected due to their involvement in the serotonin pathway (Backström and Winberg, 2017; Hoseini et al., 2017), protein synthesis and degradation pathways and modulation of antioxidant activity (Zhao et al., 2019).

Trp is degraded by tryptophan hydroxylase (TPH), which is responsible for 5-HT synthesis. There are four TPH isoforms in zebrafish: TPH1a and TPH1b, TPH2 and TPH3, which can be mainly found in diencephalic and peripheral 5-HT cells, the raphe and hypothalamic areas, respectively (Winberg and Thörnqvist, 2016). Serotonin transporter genes are important in modulating of 5-HT signaling, reducing 5-HT concentrations, and stopping synaptic activity. As a result of genomic duplication, teleosts show multiple paralogous genes. Paralogs genes can have divergent expression, as it has been reported for 5-HT_{1A} and 5-HT_{1B}. (Backström and Winberg, 2017). Zebrafish possesses two paralogs serotonin re-uptake transporters Slc6a4a and Slc6a4b (previously named SERTa and SERTb) (Norton et al., 2008). In zebrafish three serotonin receptors subtypes have been identified: 5-$HT_{1'}$ 5-HT_2 and 5-HT_7, three subgroups (5-HT_{1aa}, 5-HT_{1ab}, 5-HT_{1bd}) for 5-HT_1 and two subgroups for 5-HT_2 (5-HT_{2a} and 5-HT_{2c}). Serotonin receptors can be expressed in other tissues aside from the brain (Prasad et al., 2015).

The growth hormone/insulin growth like-factor hormone (GH/IGF) system has been reported as a regulator of protein synthesis through the target of rapamycin (TOR) pathway. TOR plays an important role in protein synthesis, being activated by an amino acid pool (Tang et al., 2013). Protein degradation pathways are important to eliminate non-functional proteins and to assess protein turnover, and they are controlled by IGFs (Rolland et al, 2015). The ubiquitin proteosomase pathway (UPP) is carried out by three enzymes: ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3) (Kobayashi et al., 2004, 2006; Meyer-Schwesinger, 2019). IGF1 is mainly expressed in the liver. This organ plays an important

role in amino acid metabolism as well as synthesis and exportation of proteins (Rolland et al., 2015).

The Nuclear factor (erythroid-derived 2) like 2 (Nrf2) is a component of the endogenous antioxidant defense system, and has been considered the master regulator of the cellular defense mechanism. Kelch-like ECH-associated protein 1 (Keap1) mediates the ubiquitin-dependent turnover of Nrf2, being Nrf2 inhibitor. Keap1 negatively regulates Nrf2 promoting its proteosomal degradation on the cytoplasm. Nonetheless, upon oxidative or electrophilic stimuli as well as presence in ROS, Nrf2 is released from Keap1 and induces the transcription of antioxidant enzymes (Mukhopadhyay et al., 2015, Shaw et al., 2018).

Aim:

Thus, this study aims to assess the potential effects of dietary Trp levels on growth performance and feed utilization as well as behavioral and biochemical parameters and gene expression of zebrafish juveniles.

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Chapter Two

Material and methods

Experimental Diets

Five experimental diets were formulated to be isoproteic (40%DM) and isolipidic (8% DM), including a Trp0 diet (with no Trp supplementation) and four other diets containing graded levels of Trp, with total dietary Trp of 0.19, 0.38, 0.76, 1.52, 3.04% of DM (diets Trp0, Trp2, Trp4, Trp8, Trp16, respectively). Trp was included in the experimental diets at the expense of a mixture of non-essential amino acids, and in order to avoid leaching and to delay absorption from the digestive tract, crystalline amino acids were coated with agar before mixing with other ingredients. Agar was dissolved in boiling distilled water and cooled at 40 °C before being added to other ingredients. To produce diets, grounded ingredients were mixed thoroughly and then cod liver oil and water were added to form a moist blend. This blend was then pelletized using a grinder and pellets were dried at 60ºC for 24 hours. Afterwards, pellets were crushed and sieved through a battery of sieves to obtain 200-400, 400-600, 600-1000 µm of diameter. Diet formulation and proximate composition are shown in Table 1.

Table 1 Composition and proximate analysis of the experimental diets

	Trp0	Trp2	Trp4	Trp8	Trp16
Ingredients (% DM)					
Fish meal[1]	16.00	16.00	16.00	16.00	16.00
Gelatin[2]	14.45	14.45	14.45	14.45	14.45
Corn gluten[3]	15.54	15.54	15.54	15.54	15.54
Cod Liver Oil[4]	6.06	6.06	6.06	6.06	6.06
Starch	38.75	38.76	38.79	38.85	38.96
Agar	1.00	1.00	1.00	1.00	1.00
Vitamin premix[5]	1.00	1.00	1.00	1.00	1.00
Choline chloride (50%)	0.50	0.50	0.50	0.50	0.50
Mineral premix[6]	1.00	1.00	1.00	1.00	1.00
Binder[7]	1.00	1.00	1.00	1.00	1.00
Lysine[8]	0.90	0.90	0.90	0.90	0.90
Methionine[8]	0.59	0.59	0.59	0.59	0.59
Tryptophan [8]	—	0.20	0.60	1.40	3.00
NEAA[9]	3.21	2.99	2.56	1.71	0.00
Proximate analysis (% DM)					
Dry matter (%)	91.06	91.02	91.28	91.33	91.93
Crude Protein	43.19	43.72	43.76	42.16	42.85
Crude Lipid	7.64	8.37	8.67	9.62	8.16
Ash	5.34	5.86	5.07	5.25	5.15
Gross Energy(kJ g^{-1})	21.32	21.87	22.07	21.82	21.22

[1]Fishmeal, Pesquera Diamante, Steam Dried, LT, Chile (CP: 74.2%; CL 10.1%) l Sorgal, S.A. Ovar, Portugal

[2] Gelatin, Sigma Type A From Porcine Skin G-1644, St. Louis, MO, USA

[3] Cod liver Oil Fagron Iberica, S.A.U, Barcelona, Spain

[4] Corn gluten (CP: 68.3%; CL: 2.9%) Sorgal, S.A. Ovar, Portugal

[5]Vitamins (mg kg^{-1} diet): retinol, 18000 (IU kg^{-1}diet); calciferol, 2000 (IU kg^{-1}diet); alphatocopherol, 35; menadionsodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinicacid, 200; pyridoxine, 5; folicacid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbylmonophosphate, 50; inositol, 400

[6]Minerals (mg kg^{-1} diet): cobaltsulphate, 1.91; coppersulphate, 19.6; ironsulphate, 200; sodiumfluoride, 2.21; potassiumiodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalciumphosphate, 8.02 (g kg^{-1} diet); potassiumchloride, 1.15 (g kg^{-1} diet); sodiumchloride, 0.4 (g kg^{-1} diet).

[7] Liptosa, Liptosa Toledo, Spain, S.A.

[8]Feed-graded amino acids: Sorgal, S.A Ovar, Portugal

[9]Non-essential amino acids mixture (% mixture): L- aspartic acid (19,47%), Glutamic Acid (29,71%), L-Serine (10,66%), L-Glycine (15,98%), L-alanine (14,14%) L- Proline (10,04%) Fagron, Barcelona, Spain

Fish rearing

Rearing System

The experimental trial took place at the Department of Biology of Faculty of Science, University of Porto, using a recirculating aquatic system (RAS) presented in Figure 5, similar to Charlon and Bergot (1984). It was composed by twenty experimental units (plastic tanks) with 10L capacity and a continuous water flow at a controlled rate. Water quality was assured by a biofilter, to maintain residual levels of ammonia and nitrite. To compensate for water evaporation and losses during tank cleaning, dechlorinated tap water at the same temperature as the system water was added to the system every day. Water temperature in the system was kept at 28±1ºC and the photoperiod was set at 14h light/10h dark.

Figure 5 Recirculating Aquatic System used in the experimental trial

Experimental Fish

Experimental fish were obtained from a laboratory-reared zebrafish broodstock. Fish were placed at a 2 male: 1 female ratio in 4 L tanks with aeration, with photoperiod was adjusted at 14h light/10h darkness and temperature at 28 ±1°C. Marbles were placed over a plastic net to serve as subtract to induce spawning. On the following morning, two hours after the start of the light cycle, eggs were collected and placed in 2L round bottom flasks and kept under the same conditions until hatching. Dead embryos were removed and water was changed daily to avoid deterioration of water quality.

Larvae were reared in the same RAS, with the previously described conditions. After yolk-sac complete resorption, larvae were fed a commercial microparticulate feed from Gemma Micro 75 (59% protein, 14% lipids, 0.2% fiber, 14% ash; package information) and *Artemia nauplii*. Food was provided, four times per day until 10dpf, and twice a day, supplemented with *Artemia* every other day, afterwards and until 51dpf.

Decapsulation of *Artemia* cysts and incubation of decapsulated cysts

Artemia spp. cysts were decapsulated by a method proposed by Sorgeloos et al. (1977), in which cysts were hydrated with seawater for 90 minutes and then collected by filtration and placed in 100mL of a buffer solution (6mL of NaOH 40% and 94mL of seawater). Afterwards, 200mL of refrigerated commercial bleach was added and cysts were kept under agitation for 2 to 4 minutes. After collection by filtration, cysts were washed abundantly with tap water and placed in a 0.6% acetic acid solution for 1 minute under agitation. Finally, cysts were collected by filtration, washed and once again washed abundantly with tap water, and at 4ºC in a 15% NaCl solution until use.

Incubation was carried out in cylinder conical containers with synthetic seawater (obtained by dissolving, at room temperature, 175g of commercial sea salt, in 5L of tap water under agitation) under continuous lighting, intense aeration from the bottom and at a temperature of about 20 ± 1 ºC for 24 hours. After hatching, the nauplii were collected through filtration with a net and thoroughly washed with system water before using as food.

Growth trial

At day 52, 400 zebrafish with an average body weight of 65.9 mg and an average length of 17.7 mm were randomly distributed into 20 groups of 20 zebrafish. Diets were randomly assigned to quadruplicate groups. Fish were fed twice a day, six days a week to apparent visual satiation, throughout the experimental period of 42 days. Utmost care was taken to assure that all feed supplied was consumed. Each food container was numbered, to avoid any bias during feeding. Food particle size was adjusted to mouth opening as fish grew: 400-600µm from 52dpf to 67 dpf and 600-1000µm from 68dpf to 94dpf.

At the beginning of the trial and every two weeks, fish in each tank were bulk weighed following 1 day of feed deprivation; also, feed containers were weighed to calculate food consumption. Fish weighing was taken with fish placed inside a beaker using system water.

Sampling

At the beginning of the trial, 100 fish from the initial stock were euthanized with excess of phenoxyethanol and frozen at -20 ºC until analysis of body composition was performed. Similarly, at the end of the trial 12 fish from each tank were euthanized using the aforementioned method and kept at -20ºC until body composition analysis was performed.

Six fish from each replicate of diets Trp0, Trp4 and Trp16 were euthanized by submersion in ice-cold water, and after the opercular movement ceased, the head was removed to extract the brain. Muscle was also collected from the flank in the three of these fish. Brains and muscle from three fish were kept at -80ºC for later determination of acetylcholinesterase activity. Brains from the other three fish were kept in perchloric acid for 2 h at 4ºC and then frozen in the perchloric acid at -80ºC until processing for determination of serotonin level. From these three fish, liver was also collected and kept in RNAlater for 24h at 4ºC and then stored at -80ºC until gene expression analysis Remaining fish were kept alive and fed as aforementioned until locomotor behavior analysis was performed.

Chemical Analysis

Chemical composition of diets and whole fish were analyzed using the standard methodology: dry matter by drying at 105°C until constant weight; crude protein by using the Kjeldahl method, following an acidic digestion and distillation (Tecator System, Högamäs, Sweden; extraction unit model 1015 and 1026, respectively); ash by incinerating samples for 16 hours at 450 °C; gross energy through direct combustion using an adiabatic bomb calorimeter. Diet lipid content was determined in a SoxTec System (Velp Scientífica SER 148/6 Solvent Extractor) using petroleum ether extraction.

Dietary amino acid content was analyzed in duplicate using high-performance liquid chromatography (HPLC), according to the Pico Tag method. Samples were hydrolyzed for 23 h with 6N hydrochloric acid at 110°C under N_2 atmosphere, and derivatized with phenylisothiocyanate reagent (PITC, Waters WAT088120) before separation by gradient exchange chromatography at 46°C (Waters auto sample model 717 plus, Waters binary pump model 1525, Waters dual absorbance detector model 2487). For an internal standard, it was used Norleucine. Chromatographic peaks obtained were identified, integrated and quantified by comparison to known amino acid standard, using Waters Breeze software package (Pierce NC10180). Tryptophan content was measured using a spectophotometric method as described by DeVries et al., 1980 (Appendix 1). Amino acid composition is shown in Table 2.

Table 2 Amino acid composition (%DM) of the experimental diets[1].

	Trp0	Trp2	Trp4	Trp8	Trp16
Arginine	2.47	2.49	2.49	2.26	2.41
Histidine	1.03	1.06	1.29	1.14	1.00
Isoleucine	1.39	1.37	1.57	1.24	1.29
Leucine	3.45	3.57	3.59	3.46	3.36
Lysine	2.87	2.84	2.95	2.74	2.77
Threonine	1.26	1.29	1.33	1.26	1.19
Valine	1.45	1.46	1.56	1.46	1.42
Methionine	1.34	1.36	1.40	1.31	1.34
Phenylalanine	1.57	1.40	1.55	1.48	1.54
Tyrosine	0.98	1.00	0.95	0.94	0.95
Tryptophan	0.22	0.40	0.91	2.02	3.34
Aspartic Acid	3.83	3.98	3.89	3.79	3.82
Glutamic Acid	6.22	6.35	6.28	6.10	6.28
Serine	2.28	2.30	1.83	1.75	1.53
Glycine	4.44	4.64	4.43	4.17	3.55
Alanine	3.93	3.91	3.87	3.68	3.64
Proline	4.48	4.32	3.89	3.59	3.37

Assessment of brain serotonin levels

Brain serotonin levels were determined at the Institute of Pharmacology and Therapeutics of Faculty of Medicine of University of Porto by High Performance Liquid Chromatography with electrochemical detection (HPLC-ECD) as described by Soares-da-Silva et al. (1995). For this method, samples were kept in perchloric acid at 0.2M for 2 hours at 4ºC and then frozen in the acid at -80ºC until processing; aliquots of the resulting solution were centrifuged at 2700 rpm for 2 min at 4ºC using Costar Spin-X microfilter tubes.

Fifty µL of the supernatant was directly injected into HPLC-ECD system. 3,4-hydroxybenzylamine (DHBA) was used as an internal standard. Lower limit for detection of 5-HT range from 350-1000 fmol. The system is composed by a pump (Gilson model 302) connected to a Manometric module (Gilson model 802C) and a stainless-steel 5-µm ODS column (Biophase Bioanalytical Systems) with 25cm length; samples were injected by means of automatic sample injector (Gilson model 231) connected to a Gilson dilutor (model 401). Electrochemical detection was performed with a glassy carbon electrode, an Ag/AgCl reference electrode and an amperometric detector (Gilson model 141), which operated at 0.75V. The procedure was monitored with Gilson 712 HPLC software.

Determination of acetylcholinesterase activity in brain and muscle

AChE activity was measured in brain and muscle samples according to a modified version of the Ellman method (1961). In brief, samples were homogenized and centrifuged at 6000xg, supernatant was recovered and used for acetylcholinesterase activity measurements. The activity of this enzyme is measured by absorbance at 412 nm due to the reaction of thiocholine and DNTB. Microplate determinations were performed on a Bio Tek Power Wave 340 reader. Brain and muscle protein content were determined, using an adaptation of the Bradford method (Bradford, 1976) for microplate. Bovine γ-globulin (1mg/ml) was used as a standard.

Locomotor Behavior

Experimental system

The experimental system was adapted from Oliva Teles et al. (2015). There were four separate recording areas, each area containing 12 arenas (plastic cups), with a total of 48 arenas being recorded each time. Each arena contained only one fish. Digital recordings were performed using four Flow Eletronics 540 IR (model: IRCAM363ULTRA) cameras. Data was stored on an Intel® Pentinum® Dual CPU (2:00 E2180@2.00GHz, 1,87 GB RAM) computer with Windows XP. Capturing software used was DSS1000 2004 version 4.7.0041. Cameras were placed above the fish to capture their movement.

In this work, we used a two-dimensional (2D) monitoring which means cameras were used to capture top view of the arenas, and thus observing movement trajectories. For trajectory to be recognized coordinates must be established in temporal sequence, in order to improve monitoring light condition must be controlled, to minimize image noise that could occur due to variation in illumination (Xia et al, 2018). This allows the determination of spatial variables that could not be evaluated in any other way, such as distance and mean velocity (Spink *et al.*, 2001).

Experimental Plan

Three trials were conducted, each having the duration of 45 min, consisting of a 15 minute acclimatization period (Gerlai et al., 2009) and 30 minute recording. Twelve fish from each replicate of diets Trp0, Trp4 and Trp16 were used on a total of 144 fish filmed at the end of the three trials. Throughout the trial fish were kept unfed. Distribution of conditions was randomized and trials were conducted between 10 am and 4 pm as it is the time in which fish are more active (MacPhail et al., 2009). Each arena contained 200ml of system water and all arenas were placed to be centered off the camera to avoid poor tracking in peripheral areas.

Video Processing

The purpose of processing the video was to individualize each fish transforming its location into XY coordinates. Firstly, each video recording must be transformed into a series of individual images (frames), this task was performed using *VirtualDub*, which converts the AVI files into ImageSequence. ImageJ software was used to individualize each experimental condition, recognizing each fish as a "spot", a black dot on a white background. Each dot tracked means a set of coordinates, and the coordinates for each frame are then exported to an Excel spreadsheet. Each frame needs to be converted into 8-bit grayscale, and background subtracted using the tool *Rolling Ball Background* with a light background filter, allowing us to eliminate shadows and small interferences enabling contrast from animals (Figure 6).

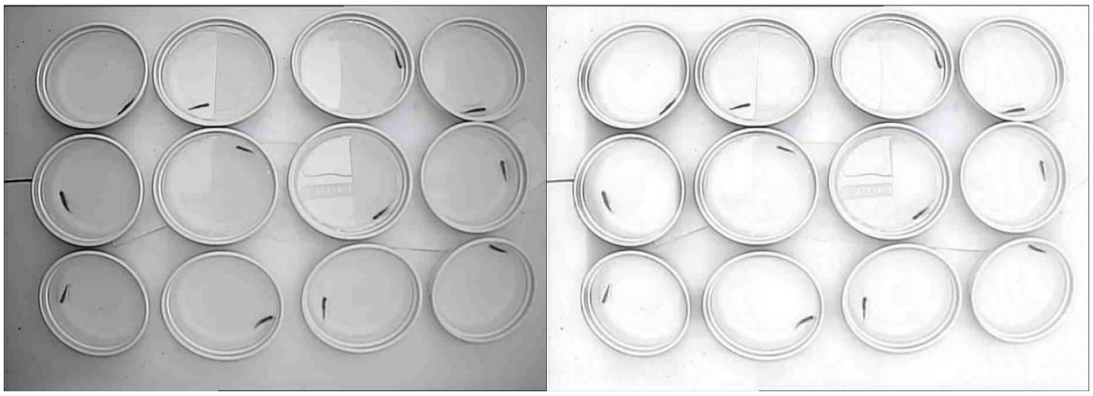


Figure 6 Before and after using the Rolling Ball Background Tool on ImageJ, with the light filter background

Each arena was selected using the Oval tool and individualized because the tool Multitracker works better when it follows only one fish at a time. (Figure 7).

Figure 7 Before and after the selection of the arena using ImageJ

Using the adjusting tool *Threshold* it was possible to convert each fish into a black spot (Figure 8). Lastly, *MultiTracker* plugin (version 2001/7/17(Jeffrey Kuhn, University of Texas at Austin)) was used to recognize the coordinates. Having transformed the location into XY coordinates it is possible to calculate movements used for statistical analysis.

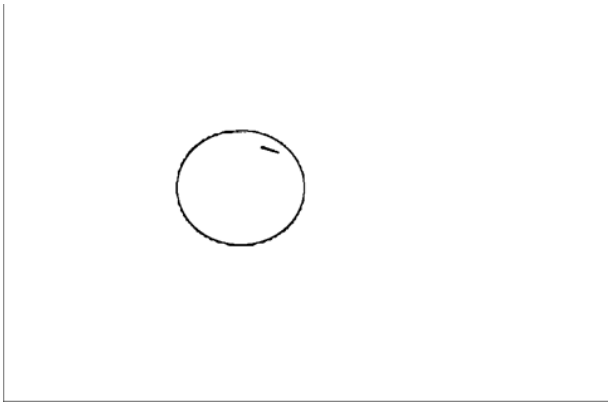


Figure 8 Using the tool Threshold in ImageJ

Gene expression in liver:

RNA extraction and cDNA synthesis

RNA was extracted from pools of 3 livers using the Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare). Six pools were evaluated for each diet tested. A digestion of genomic DNA was subsequently carried out using deoxyribonuclease I Amplification Grade. Quantification of RNA was performed with 2µL of eluate using a Take3 micro-volume plates in BioTek microplate spectrophotometer, according to the micro-volume quantification method. Then, 1µg of RNA was used to synthesize cDNA, with the qScript cDNA Synthesis Kit (Quanta BioSciences, Inc.).

Quantitative Real Time Polymerase Chain Reaction (qPCR)

Three reference genes and twelve genes of interest were assessed in zebrafish liver (Table 3). The name and pairs of primers (forward (F) and reverse (R)) used as well as their respective efficiency are also presented in Table 3. Primers for UBE2H, TOR, Nrf2, Keap1, and reference genes were obtained from literature available for zebrafish (Table 3). For the remaining genes of interest, the primers were designed using gene sequences available in GenBank, using the Pick Primers tool from the National Centre of Biotechnology Information (NCBI).

In order to evaluate gene expression, reverse transcriptase quantitative real time-polymerase chain reaction (qPCR) was performed in an Eppendorf Mastercycler realplex 4 (Eppendorf, Hamburg, Germany). The qPCR assays were performed with a final volume of 20µL, using 10µL of Sybr® Green SuperMix (Quanta BioSciences, Inc.), 4µL of water, 2µL of F primer, 2µL of R primer, and 2µL of cDNA. Reactions were performed in duplicates as follows: 2 minutes of denaturation at 94ºC; 40 cycles of denaturation for 30 seconds at 94ºC, 30 seconds at 54ºC for annealing (for reference genes, TOR, UBE2H, Htr1aa and Htc2cl1, Slc6a4a, Keap1, Nrf2, TPH1a, TPH1b), at 52ºC (TPH2a, TPH2b), at 59ºC (IGF1), and 30 seconds at 72ºC for elongation. A final elongation was also performed for 10 min at 72ºC. A blank sample was done for each gene in order to identify possible nonspecific products.

Standard linear curves were made for all primers, in order to determine the efficiency of each primer; eight dilutions were used from 0.5 to 50 ng/µL, from an initial mixture of cDNA. Quantification of gene expression was done with normalization by reference genes using the NormFinder algorithm (Urbatzka et al., 2013). Relative expression using the overall mean of all samples as calibrator and accounting for efficiency, using the mathematical template of Plaffl (Plaffl, 2001)

Table 3 GenBank accession numbers, primer sequences concentrations, amplicon lengths and efficiency of reaction and function in the organism for target genes investigated in the present study

Assessed gene	Function	Accession number	Primers	Efficiency (%)
UBE2H[1]	Ubiquitin-conjugating enzyme E2H	NM_201489.1	F:GGACCACAAGGAACACCGTA R: TAAACACAGGTCCCTGACG	113
TOR[2]	Target of rapamycin	BC091880	F:CGCACTGATTCGAGACTACAG R:TCAAACACTTCCACCTTCTCC	109
IGF1	Insulin growth-like factor 1	AF268051	F: GGCAAATCTCCACGATCTCTA R:CGGTTTCTCTTGTCTCTCTCA	110
Nrf2[3]	Nuclear factor (erythroid-derived 2)-like 2	NM_182889.1	F: TGGCCCTGAAGAATTTAACG R: CCCGGTGAGAAGCTCTGTAG	108
Keap1[3]	Kelch-like ECH- associated protein 1	NM_182864.2	F: TGATGGACAAACCCAACTCA R: CACTGGACAGGAAACCACCT	117
TPH1a	Tryptophan hydroxylase 1 a	NM_178306.3	F: CGACAGCAACCGTGAACAAC R: GGCACATTCTCCATCTCGC	100
TPH1b	Tryptophan hydroxylase 1 b	NM_001001843.2	F: CTAAGAGCATACGGGGCTGG R :GGGCTCAAAGGGCAGGATTT	96
TPH2a	Tryptophan hydroxylase 2 (variant 1)	NM_001310068.1	F:GTATGACCAACAGCACCTTG R: TCACCAGAAAGCCAACTTCA	123
TPH2b	Tryptophan hydroxylase 2 (variant 2)	NM_214795.2	F:GTATGACCAACAGCACCTTG	112

			R:TCACCAGAAAGCCAACTTCA	
Htr1aa	Serotonin receptor	NM_001123321.1	F:ATGAGGATGAGCGGGATGTAG R:CAATCAGCCAGGACCACG	125
Htr2cl1	Serotonin receptor	NM_001129893.1	F:GCGCTCTCTGTCCTATTTGG R:GTAGCGGTCGAGAAATGG	126
Slc6a4a	Serotonin transporter	NM_001039972	F: CATCTATGCTGAGGCTATTG R:AAGAATATGATGGCGAAGA	100
Ef1[4]	Reference Gene	NM_131263.1	F: GGACACAGAGACTTCATCAAGAAC R: ACCAACACCAGCAGCAAGT	117
Actb1[4]	Reference gene	NM_131031.1	F: TCCCAAAGCCAACAGAGAGAAG R: GTACACCATCACCAGAGTCC	101
Rpl8[4]	Reference gene	NM_200713.1	F: CAATGACGACCCGACCG R: CGCCAGCAACTCAGTCACT	96

Primers were obtained from:

[1] Dhanasiri et al. (2013)

[2] Craig et al. (2011)

[3] Mukhopadhyay et al (2015); Shaw et al. (2018)

[4] Cunha et al., (2018)

Statistical Analysis

Data collected during the experimental trial (initial and final fish weight, feed consumption, chemical composition of diets and initial and final fish body chemical composition) were used to determine growth performance and feed utilization indices, including Weight Gain (WG), Feed Efficiency (FE), Protein Efficiency Ratio (PER), Daily Growth Index (DGI), Nitrogen Intake (NI), Nitrogen Retention (NR), Energy Intake (EI) and Energy Retention (ER). Data are presented as means and pooled standard error of mean (SEM).

Polynomial Orthogonal Contrasts analysis was performed to determine whether the data followed linear, quadratic and/or cubic responses to the dietary graded levels of Trp, significance was accepted when $p<0.05$. This statistical analysis was performed using IBM SPSS Statistics 25.0 software package for Windows.

Gene expression analysis was performed by means of one-way Analysis of Variance (ANOVA), significance was accepted when $p<0.05$. Linear contrasts were also applied to ANOVA when applicable. Data were transformed when needed to meet ANOVA requirements. When ANOVA requirements could not be met a non-parametric test (Krusktal-Wallis) was performed to investigate possible differences between dietary treatments. Differences between the expression levels of genes within diets were sought using the *t*-test for paired samples or the non-parametic Wilcoxon test for paired samples. Correlations between gene expressions were calculated using the Pearson's correlation coefficient. A Heatmap was generated on the Pearson's correlation matrix with Factoextra R Package (R Core Team, 2014).

Discriminant Analysis was used to integrate all gene expression data. The dependent variable (categorical variable) entered in the model described the three tested diets: non-supplemented, moderately and highly supplemented diets for Trp0, Trp4, Trp16, respectively. Predictors entered in the model were the twelve assessed genes and two by two combinations, to investigate possible interactive responses elicited by the dietary Trp levels. Two outliers were excluded from the data and the prior category probabilities of the dependent variable were computed from this dataset. One-way ANOVA followed by Tukey HSD test was used to investigate homogenous groups and describe the contribution of each significant predictor retained in the final model to group discrimination.

For behavior analysis eight parameters were calculated including animal position (mean value for the X and the Y coordinates, in (mm), velocity (mm/s), angular velocity (degrees/s), linear acceleration (mm/s^2), angular acceleration ($degrees/s^2$), a measurement of dispersion of the fish (product of the standard deviation of XY) and mean meander (degree of curvature by unity

of displacement in degrees/mm). A descriptive analysis was performed by one-way ANOVA; when ANOVA requirements could not be met non-parametric tests were used. These analyses were performed in SPSS version 25.0 package for Windows.

A Cluster Analysis was performed in order to reduce variables to create behavioral classes used for a Correspondence Analysis. Then, behavior classes were defined by means of a Kohonen-type artificial neural network that grouped each instant (frame) of fish into four behavior classes based on previously selected variables. This analysis groups the data by their similarity, meaning that variables in the same group have similar values. One-way Analysis of Variance with the Tukey HSD was used to investigate differences among behavioral classes. A Correspondence Analysis (CA) was subsequently carried out to investigate possible behavior differences among Trp levels. Data entered in the Correspondence Analysis were the behavioral classes defined by the Cluster Analysis against the Trp levels. Homogeneous groups among Trp levels were investigated using the Chi-square test with the Bonferroni Correction. All these analyses were performed using Statistica v13 (StatSoft, 2012) for Windows.

Results

1. Growth Performance

Diets were well accepted by fish and mortality was low (less than 5%) and similar in all experimental groups. Growth performance, feed intake, feed efficiency, and protein efficiency ratio were similar among experimental diets (Table 4). Daily nitrogen retention showed a quadratic tendency with a maximum for the diet Trp4 (0.81 g kg ABW^{-1} day^{-1}). Similarly, nitrogen retention expressed in percentage of nitrogen intake showed a quadratic response, attaining a peak with diet Trp8 (24.21%NI). Daily energy retention was not affected by dietary treatment, but when expressed as percentage of energy intake, a quadratic response was observed with a peak for the diet Trp8.

Whole-body composition is presented in Table 5. Dietary treatments did not affect final whole-body composition, except for gross energy, which showed a quadratic response, with a peak at Trp8 diet (8.27 kJ g^{-1}).

Table 4 Growth performance and feed utilization of Zebrafish fed the experimental diets'.

							P-value		
Diet	Trp0	Trp2	Trp4	Trp8	Trp16	SEM	Linear	quadratic	Cubic
Initial body weight (mg)	65.40	65.50	66.88	65.63	66.13	0.00	0.469	0.593	0.965
Final body weight (mg)	251.25	252.16	245.34	240.50	248.70	0.00	0.754	0.299	0.834
Weight gain (g/kg ABW/d)[1]	27.61	27.57	28.47	26.88	27.03	0.31	0.676	0.390	1.000
Daily growth index[2]	0.54	0.54	0.52	0.52	0.53	0.00	0.603	0.369	0.832
Feed intake (g kg $ABW^{-1}day^{-1}$)[3]	51.00	47.31	49.91	46.03	47.26	0.75	0.143	0.228	0.837
Feed efficiency[4]	0.55	0.59	0.54	0.60	0.58	0.01	0.303	0.529	0.707
Protein Efficiency ratio[5]	1.27	1.35	1.23	1.37	1.35	0.03	0366	0.473	0.588
N Intake (g kg ABW^{-1} day^{-1}) [a]	3.57	3.56	3.66	3.15	3.40	0.07	0.359	0.384	0.273
N Retention (g kg ABW^{-1} day^{-1})[b]	0.71	0.74	0.82	0.75	0.76	0.01	**0.004**	**0.012**	0.196
N Retention (% NI)[c]	19.89	22.17	22.36	24.21	22.21	0.53	**0.019**	**0.025**	0.101
E Intake (kJ kg ABW^{-1} day^{-1}) [d]	1102.93	1052.15	1138.64	1015.84	1057.54	18.69	0.332	0.250	0.297
E retention (kJ kg ABW^{-1} day^{-1}) [e]	240.08	255.33	265.22	291.88	288.35	7.38	0.652	0.155	0.155
E retention (%EI)[f]	21.82	24.27	23.25	29.34	27.30	1.07	0.237	**0.037**	0.946

Values are presented as means and pooled standard error of the mean (SEM)

ABW: average body weight = (initial body weight IBW +final body weight FBW)/2

[1]WG= [(FBW-IBW)*1000)/ (IBW+FBW)/2)] × ($days^{-1}$).

[2] DGI=100 × [((final weight) $^{1/3}$- (initial weight) $^{1/3}$) x $days^{-1}$]

[3]FI= [(ingested food*1000)/ (average body weight/1000)] × ($days^{-1}$).

[4]FE = weight gain (g) / dry feed intake (g)

[5]PER: wet weight gain (g) / dry crude protein intake. (g)

[a] NI= [protein intake (g)/6.25*1000)]/ (ABW*days)

[b] NR= [((FBW*Final body protein (%)) - (IBW*initial body protein (%))/6.25*1000]/ABW*days

[c] NR (%NI) = (NR/NI)*100

[d] EI= (energy ingested*1000)/ (ABW*days)

[e] ER= [(FBW*final energy FM) – (IBW*initial energy FM)*1000]/ABW*days)

[f] ER (%EI) = (ER/EI)*100

Table 5 Body composition of fish fed the experimental diets (wet weight basis).

								p-value		
Diet	Initial Body composition	Trp0	Trp2	Trp4	Trp8	Trp16	SEM	Linear	quadratic	Cubic
Dry matter (%)	18.74	26.68	28.72	28.41	26.73	27.77	0.44	0.922	0.264	0.153
Protein	12.27	14.57	15.02	16.52	15.30	15.34	0.27	0.542	0.124	0.103
Gross Energy (kJ g^{-1})	4.89	7.46	7.80	8.19	8.27	7.54	0.13	0.959	**0.005**	0.704
Ash	9.50	6.18	4.43	4.36	3.56	4.21	0.62	0.349	0.499	0.961

Values are presented as mean and pooled standard error of the mean (SEM).

2. Brain Serotonin Levels

No significant differences were observed in brain serotonin levels between treatments, however, contrasts indicate a linear tendency, as brain 5-HT levels increase with increasing dietary Trp levels (Figure 9).

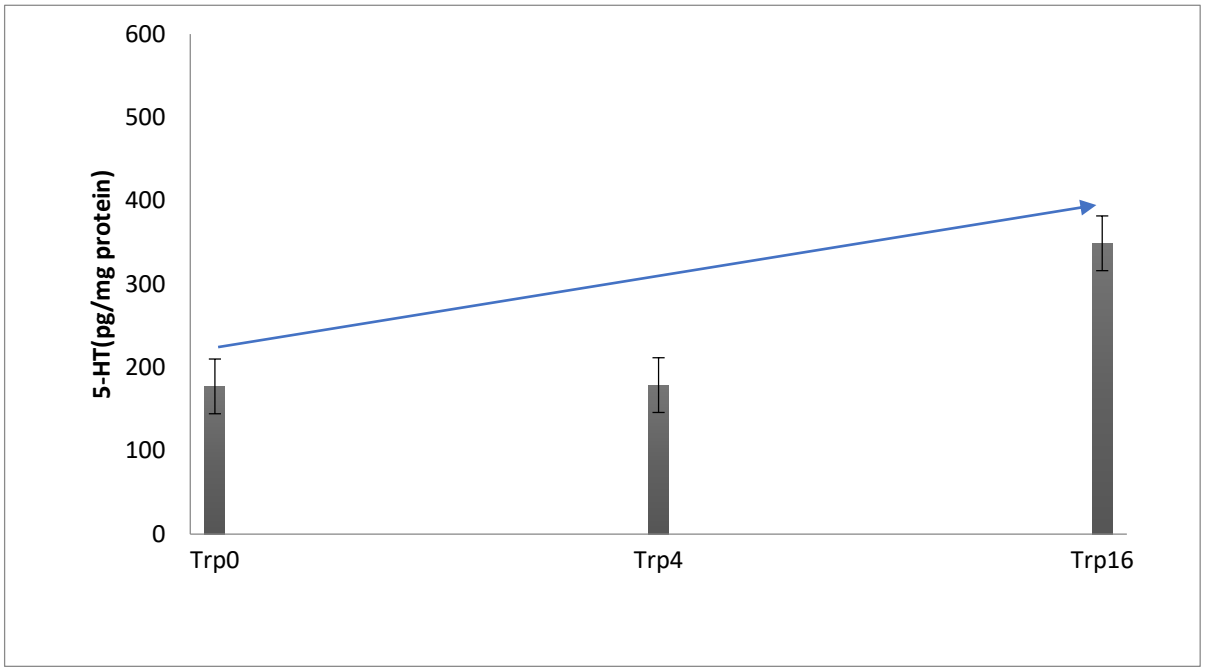


Figure 9 Whole-brain levels of serotonin (5-HT expressed in pg/mg protein). Graphic presented as means ±standard error of the mean (SEM). Blue arrow indicates linear tendency ($p<0.05$)

3. AChE activity in muscle and brain

Muscle AChE activity showed no significant differences among dietary treatment ($p>0.05$), however, brain AChE activity had a quadratic response trend as function of Trp level. Irrespective of the diet, AChE activity was two times higher in the muscle than in the brain (Table 6).

Table 6 Acetylcholinesterase activity in the brain (AChEb) and muscle (AChEm) in nmol min^{-1} mg $protein^{-1}$

					p-value	
Diet	Trp0	Trp4	Trp16	SEM	Linear	quadratic
AChEb	61.10	74.00	64.67	2.61	0.98	**0.04**
AChEm	132.41	142.93	131.90	8.62	0.87	0.64

Values are presented as mean and pooled standard error of the mean (SEM).

4. Locomotor behavior

After processing the behavioral recordings with Image J, the data obtained was first analyzed for a reduction of variables. For this, a Cluster Analysis based on a dissimilarity distance (1-Pearson *r*) was performed to identify variables showing highly similar patterns of response and, thus, redundant in the subsequent analysis (Figure 10).

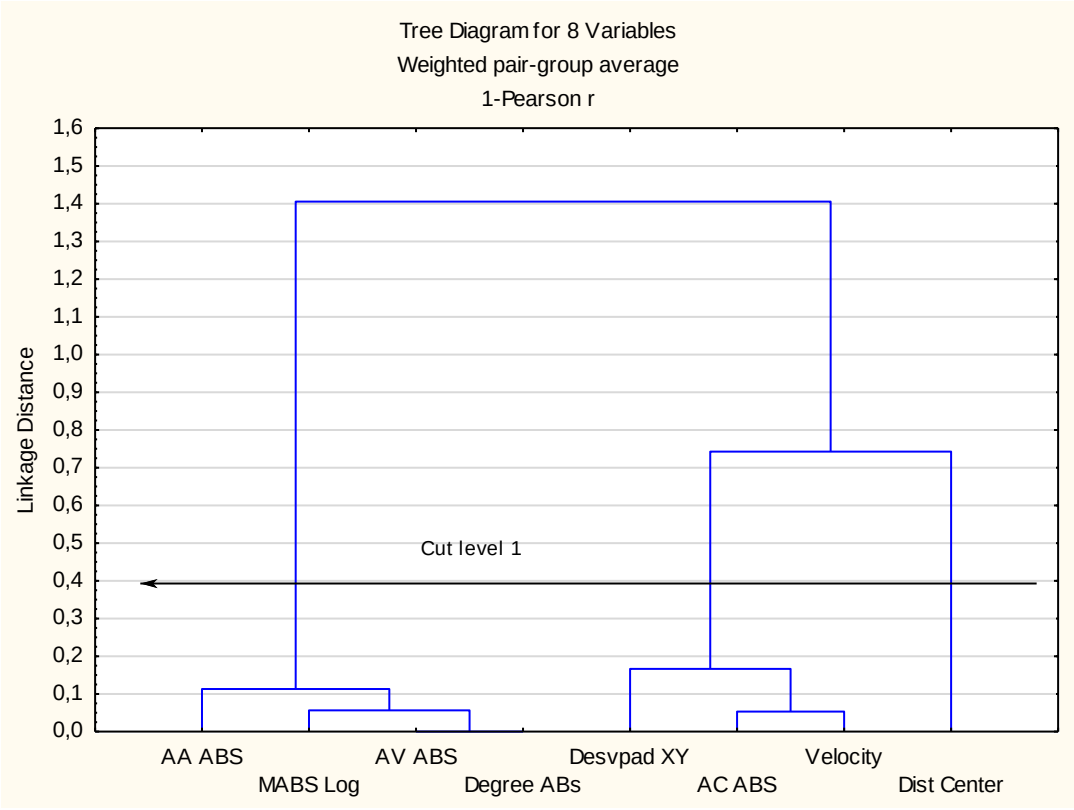


Figure 10 Cluster Analysis using eight variables; also represneted is the cut-off level. AA- angular aceleration; M – Meander; AV- Angular Velocity, Degree- degree; Desvpad XY- DesvpadX*DesvpadY; AC- acceleration, Velocity and Dist Center- Distance to the center.

A high number of variables can become difficult to work with and some may even show high similarity in pattern, presenting redundant information. In figure 10, it is possible to observe for example that meander and angular velocity, as well as acceleration and velocity, are highly related. Overall, three clusters were identified, thus, reducing information. Table 7 indicates the different variables in each cluster and the respective Chi-square values. Chi-square values were calculated from the frequency of fish in different size classes of each behavioral variables in function of Trp level.

Table 7 Groups of variables identified in the Cluster Analysis. Highlighted variables were chosen for a descriptive and Correspondence Analysis.

Cluster level	Variable	Pearson Chi-Square
a	**Meander**	**324**
	Angular acceleration	285
	Angular velocity	217
	Degree	179
b	Acceleration	586
	Velocity	**536**
	Dispersion	375
c	**Distance to the center**	**698**

From Table 7, one variable from each cluster were selected for the subsequent data analyses; meander was selected from cluster a and velocity was chosen from cluster b. Together with Distance to the center (cluster c). These three variables were used in the subsequent analysis with a neural network algorithm and the Correspondence Analysis.

Model was adjusted to four classes, meaning four behavior classes were identified by the neural network according to similarity (Table 8). All behavior classes are significantly different (Tukey HSD test) with respect to the three selected behavioral variables

Table 8 Homogenous groups defined by χ^2 test with Bonferroni correction in function of dietary Trp levels

Behavioral classes	Trp0	Trp4	Trp16	Distance to the Center	Velocity	Meander
(1, 2)	b	a	a	66,0	31,3	0,479
(2, 2)	b	c	a	63,9	15,2	0,865
(2, 1)	a	b	b	44,4	9,4	1,264
(1, 1)	a	b	c	60,8	2,5	2,366

Correspondence Analysis

A correspondence analysis was performed with the χ^2 test, thus allowing the understanding of the interaction of variables that resulted in the diagnosis. Through Figure 11, it is clear that each dietary treatment (Trp level) is associated to a different behavior class: class (1,2) is positively associated with Trp0; class (1,1) is positively associated with Trp16; class (2,2) is positively associated with diet Trp4.

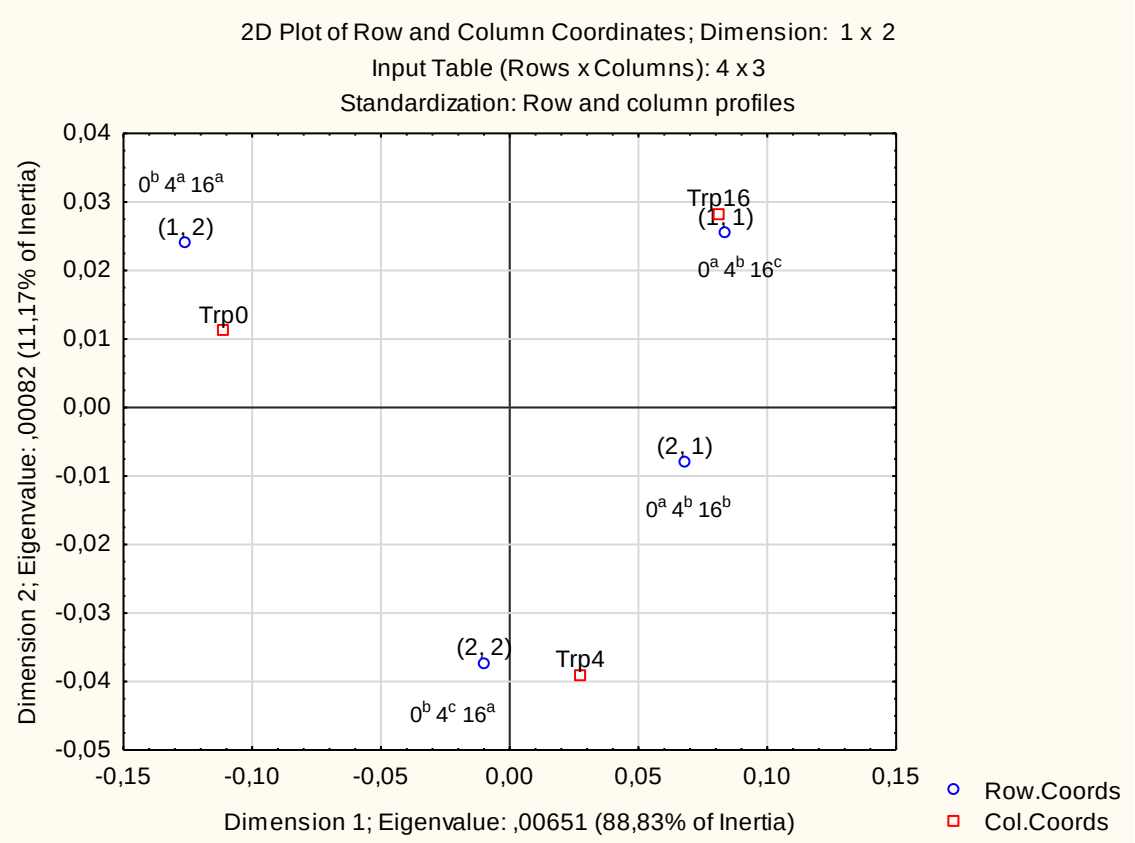


Figure 11 Statistica 2D plot, summarization of the dietary treatments and behavioral classes associated with each dietary treatment. Blue dots show coordinates for different behavioral classes. Red dots show coordinates for the dietary treatment. Numbers beneath each behavior class show dietary treatment. Small letters indicate statistical differences.

The χ^2 test showed significant differences between dietary treatments (Table 9), meaning that behavior was affected by the dietary treatment. Velocity is negatively correlated with meander. According to Table 10, behavior classes (1,2) and (1,1) had higher contributions to the χ^2 meaning these behaviors were most affected by dietary treatment, indicating some sort of disturbance. These behaviors classes are positively associated to diets Trp0 and Trp16, respectively, and contain extreme values in velocity. These behaviors have an average and elevated frequency, respectively (Table 11).

Table 9 Statistica Eigenvalues and Inertia for all Dimensions

Number of Dims.	Eigenvalues and Inertia for all Dimensions Input Table (Rows x Columns): 4 x 3 Total Inertia=,00733 Chi²=288,43 df=6 p=0,0000				
	Singular Values	Eigen-Values	Perc. of Inertia	Cumulatv Percent	Chi Squares
1	0,080715	0,006515	88,82620	88,8262	256,1993
2	0,028628	0,000820	11,17380	100,0000	32,2283

Diet Trp4 is negatively associated with the previous classes, consisting on average parameters, which can be associated with a smaller stress. However Trp4 is also correlated with Trp 16 with behavior class (2,1), which has average velocity but a smaller distance to the center, which can indicate a stress behavior; this is, however, the least frequent behavior class (11%). Moreover, diet Trp4 is associated positively to class (2,2) with average velocity and average distance to the center, corroborating the notion that fish are not so disturbed. Behavior class (2,2) is the most frequently observed (34%) with the smallest contribution to the χ^2test.

Globally, the majority of behavioral changes are observed on diets Trp0 and Trp16, contributing to 90% of the χ^2test (56% and 34% respectively), with behavior classes (1,2) and (1,1) contributing with 54% and 32%, respectively. The fact that these frequent behaviors are associated with maximum velocity (1,2) and minimum velocity (1,1), seems to indicate that fish fed on these diets are more disturbed than those fed on diet Trp4.

Table 10 Contributions to χ^2 of each dietary treatment for each behavioral class

Behavioral classes	Contributions to Chi-Square Row variables: Position2x2(4) Column variables: Concentração(3)				
	Trp0	Trp4	Trp16	Total	%
(1, 2)	102,9094	18,03062	33,54672	154,4867	57
(2, 2)	0,0050	10,19658	9,75273	19,9544	7
(1, 1)	44,3596	0,15683	48,81230	93,3287	7
(2, 1)	13,5224	1,72115	5,41425	20,6578	32
Total	160,7965	30,10519	97,52599	288,4277	100
%	56	10	34	100	

Table 11 Observed Relative frequency of each dietary treatment in each behavioral class.

Behavioral classes	Percentages of Total Row variables: Position2x2(4) Column variables: Concentração(3)			
	Trp0	Trp4	Trp16	Total
(1, 2)	9,32486	7,44819	7,22695	24,0000
(2, 2)	11,15575	11,94914	10,87349	33,9784
(1, 1)	9,06548	10,28608	11,48379	30,8353
(2, 1)	3,32104	3,88303	3,98220	11,1863
Total	32,86713	33,56643	33,56643	100,0000

5. Gene expression in liver

According to NormFinder results, the most stable gene for single use was *Actb1* (stability value: 0.017). However, the combination between A*ctb1* and R*pl8* (stability value: 0.013) was the most stable for multiple reference genes. Therefore, the normalization factor of *actb1* + *rpl8* was employed to normalize the expression of all genes.

Figure 12 illustrates the gene expression levels obtained for serotonin receptors Htr2cl1 and Htr1aa, as well as serotonin transporter Slc6a4a. Statistically, significant differences were detected for Htr2cl1 in fish fed the Trp4 diet. Differences were also encountered for Htr1aa, in animals fed both Trp4 and Trp 16 diets having a lower relative expression when compared to those fed Trp0.

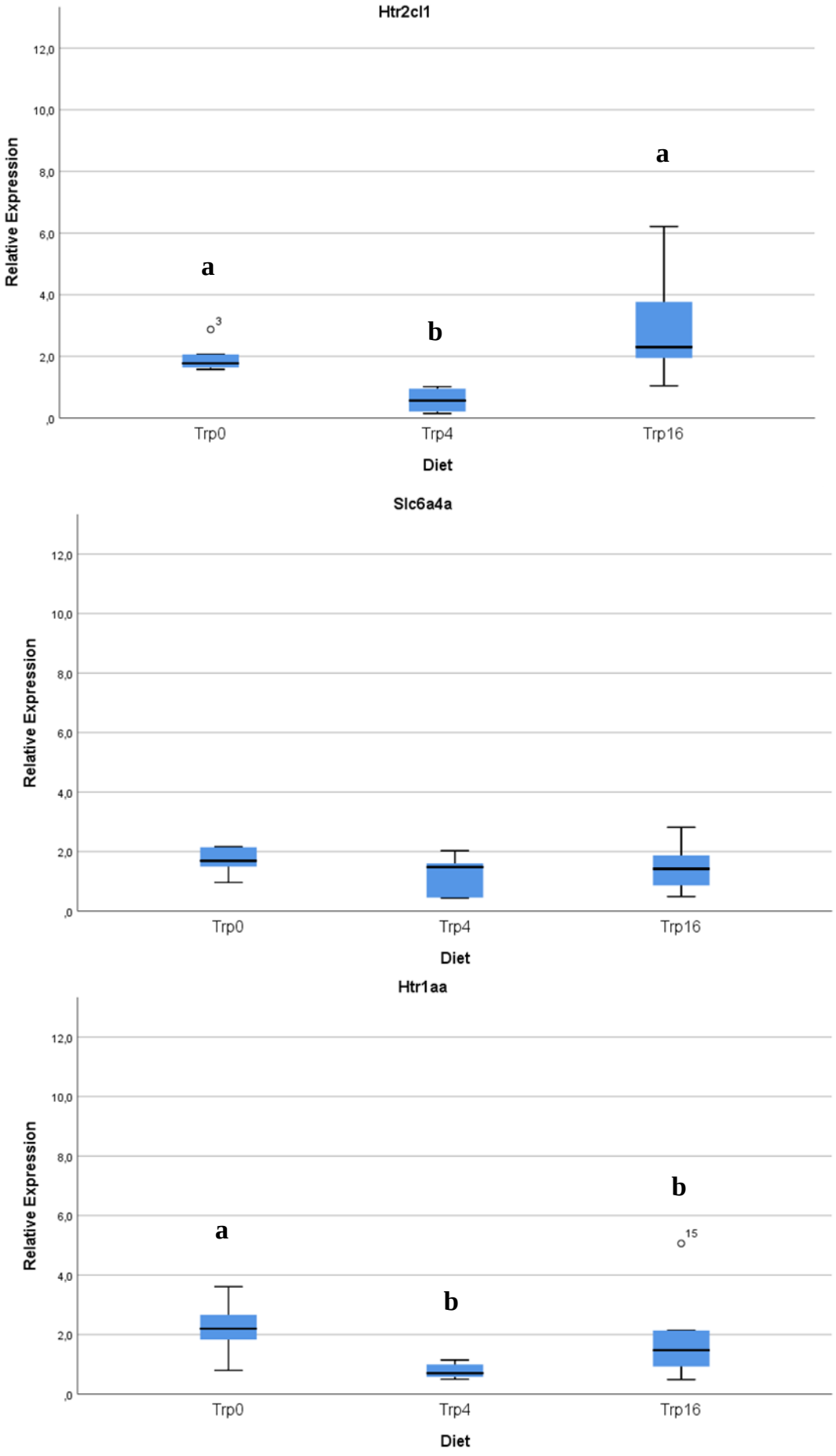


Figure 12 Relative Expression of serotonin receptors Htc2l1 and Htr1aa and serotonin transporter Slc6a4a for each dietary treatment.

Figure 13 presents the levels of gene expression for Nrf2 and its repressor Keap1. A linear tendency of increase in expression with the increase of dietary Trp was encountered for Keap1. No significant differences among diets were found for Nrf2 expression levels. Because Keap1-Nrf2 inhibitory complex is recognized as the major regulator of cellular protective responses against electrophilic stress and oxidative stress caused by reactive oxygen species (ROS), possible differences in the expression levels of these two genes within diet were also sought. The statistical analysis showed the levels of Nrf2 were similar to those of Keap1 for Trp0 and Trp4. They were, however, 29% lower than Keap1 in fish fed Trp16, suggesting that the later may be exerting its repressive effects over Nrf2.

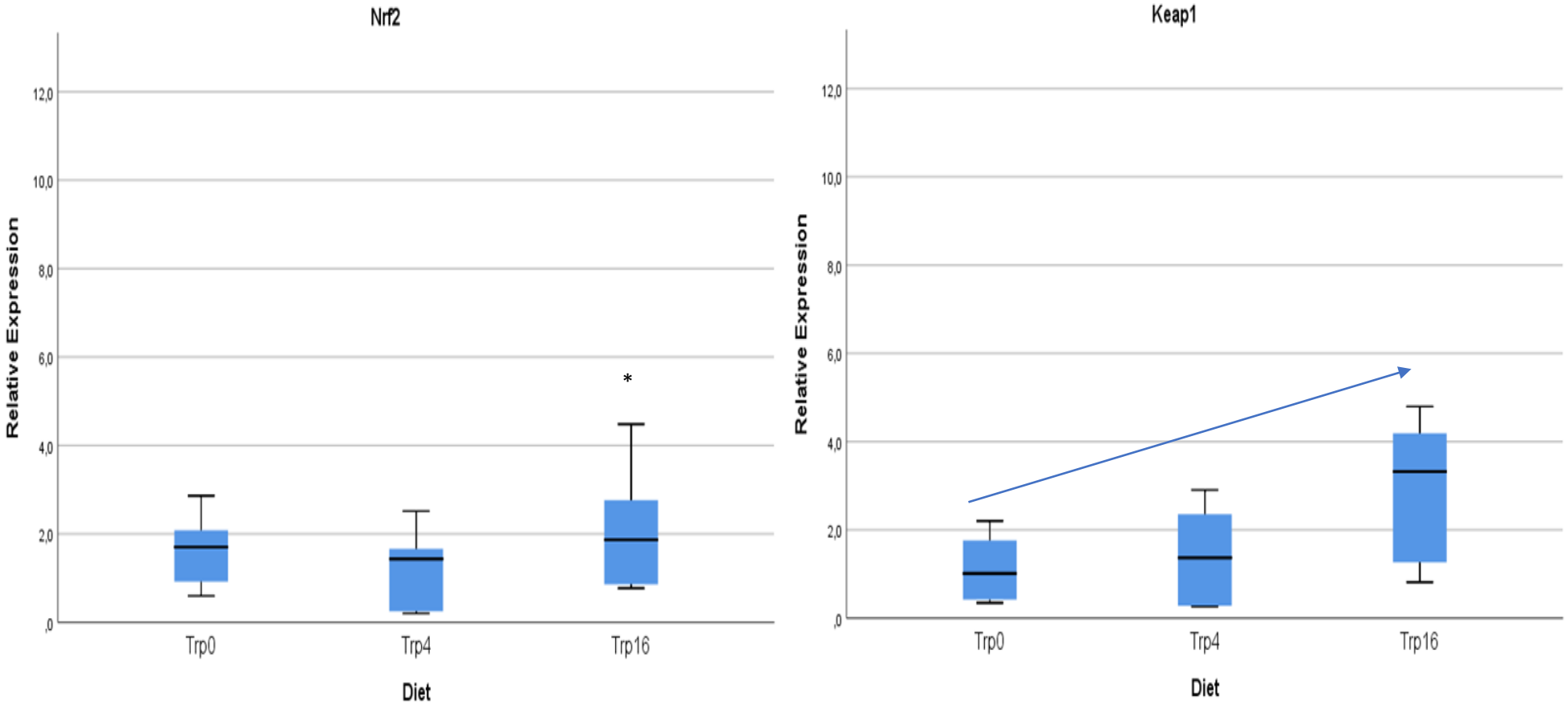


Figure 13 Relative Expression levels of Keap1 and Nrf2. Blue arrow indicates linear tendency for Keap1 gene. Asterisk represents significant differences between Nrf2 and Keap1 for the same dietary treatment (Trp16)

IGF1, TOR, and UBE2H expression levels are presented in Figure 14. No significant differences were found for the expression levels of UBE2H and IGF1 genes among dietary treatments. However, a linear tendency was encountered for TOR gene, with its expression levels increasing with the increase of dietary Trp.

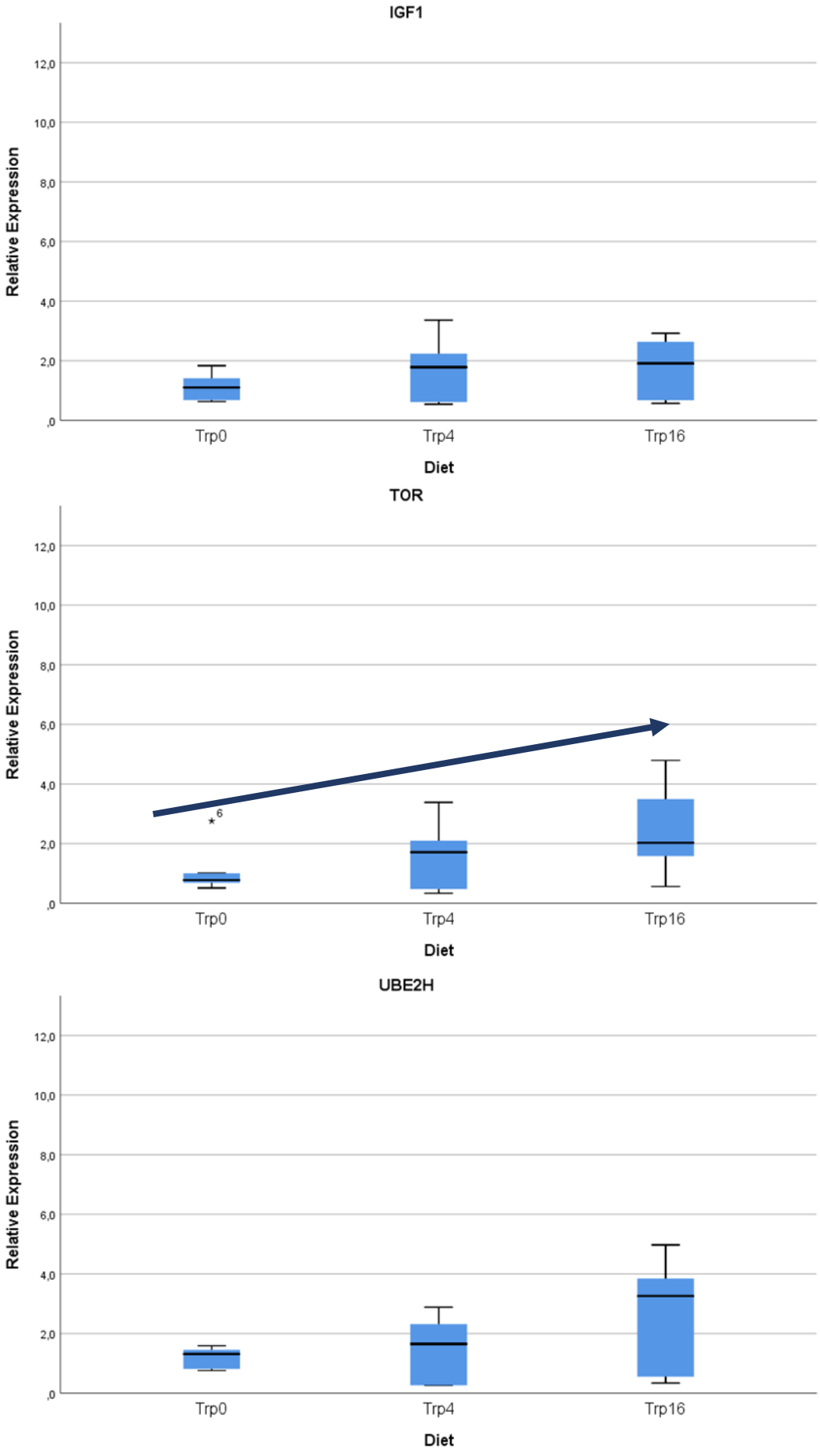


Figure 14 Relative Expression of IGF1, TOR and UBE2H. Blue arrow indicates an increasing linear tendency for TOR gene

Figure 15 shows the expression levels for two isoforms of tryptophan hydroxylase 1 (TPH1a and TPH1b) as well as the two variants of tryptophan hydroxylase 2 (TPH2a and TPH2b). No significant differences among diets were observed for the Trp degrading enzymes TPH1b and TPH2 and TPH2b. A negative linear tendency was encountered for TPH1a, showing that increasing levels of Trp decreased its expression in the liver. On diet Trp16, expression levels of Htr2cl1 is three times higher than Htr1aa, also TPH2a expression levels is 2.5 times higher than TPH2b

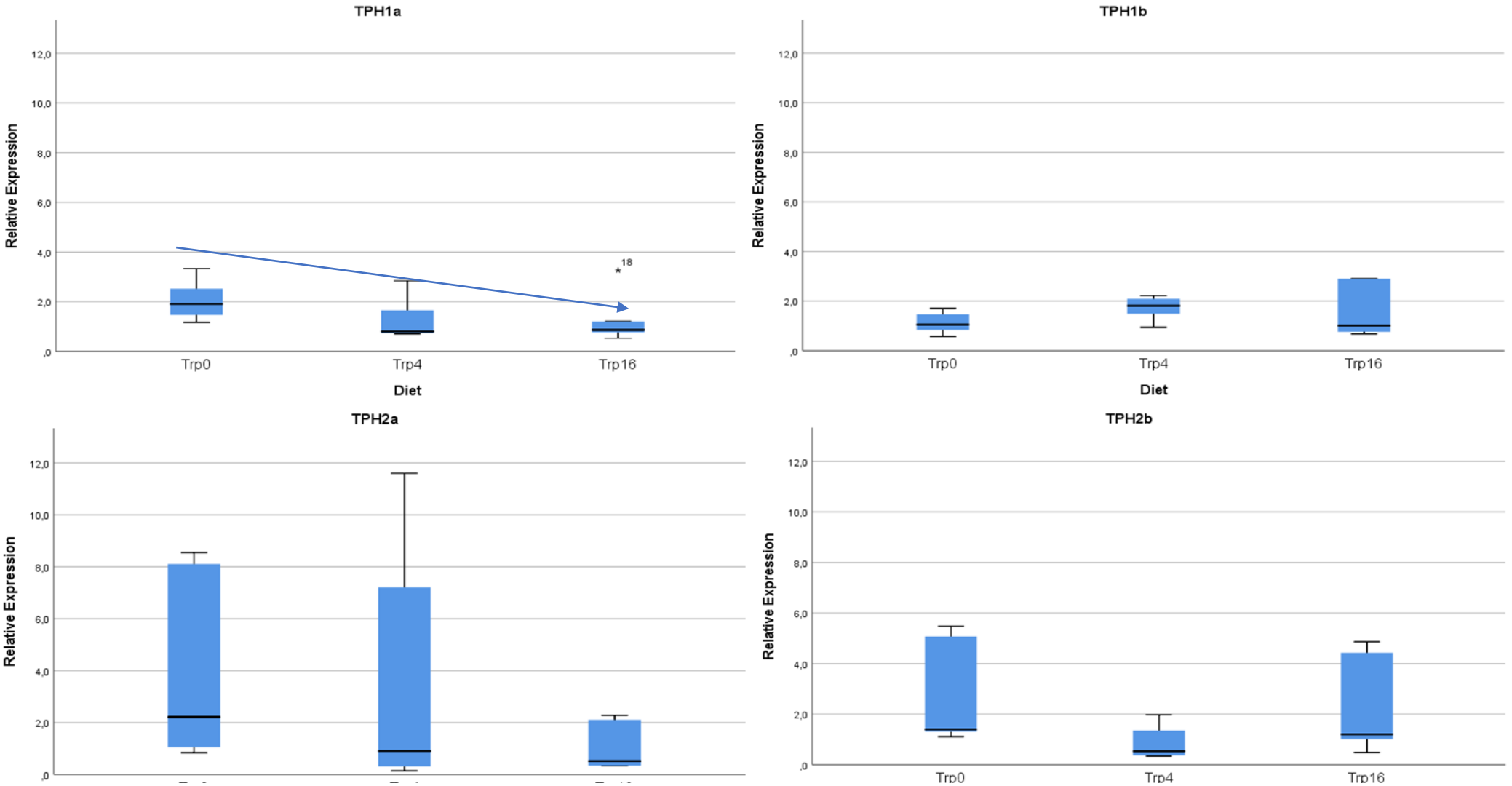


Figure 15 Relative Expression of TPH1a, TPH1b, TPH2a and TPH2b. Blue arrow indicates an increasing linear tendency for TPH1a gene

Figure 16 shows the correlation heatmap obtained for 12 studied genes. Blue color indicates positive correlations and red color indicates negative correlations, the stronger the color and the larger the size the stronger the correlation. The majority of the correlations observed between genes were positive, with one cluster exhibiting strong correlation values. This cluster is related to the UBE2H and Keap1 pathways. In fact, UBE2H was significantly correlated with Keap1 ($r=0.81$, $p<0.0001$) and IGF1 ($r=0.79$, $p<0.0001$) and to a slightly extent to Htr2cl1 ($r=0.66$, $p<0.01$) and Nrf2 ($r=0.79$, $p<0.01$). Expression of Keap1 was significantly correlated to the expression of Nrf2 ($r=0.79$, $p<0.0001$), as expected from their shared pathway, and of IGF1 ($r=0.73$, $p<0.0001$).

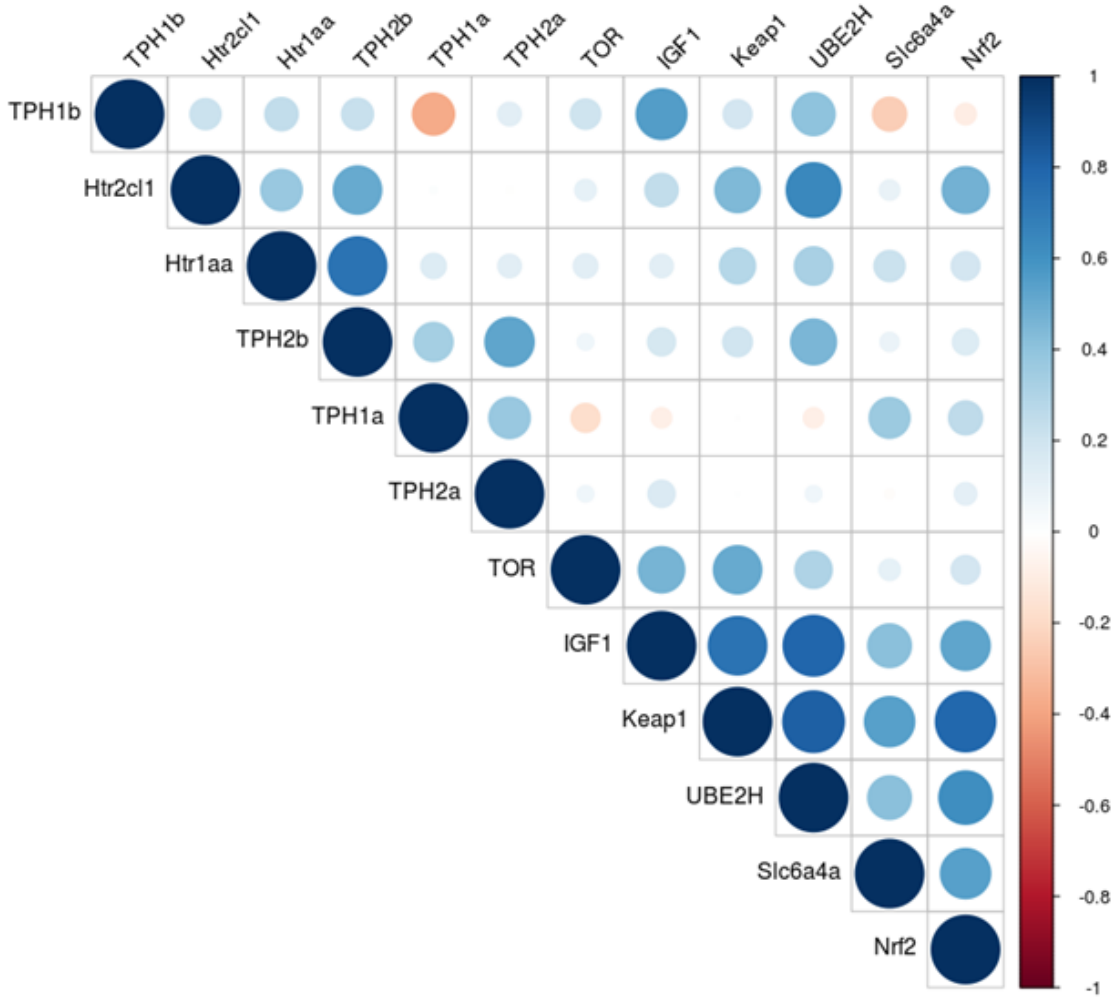


Figure 16 Correlation heatmap of relative expression of the assessed genes. Positive correlations are marked in blue, as for negative correlations are marked in red. Dark blue indicates self-correlation for gene expression.

Integrated analysis of assessed genes was done with discriminant analysis. Two discriminant functions were obtained (Table12), explaining the 85.5% and 14.5% of between group variability, respectively. The first function (horizontal) maximally discriminates the diet with Trp16 supplementation from the remaining ones (Figure17a). The second function (vertical) separated the Trp0 from the Trp4 diet (Figure 17a). Two interaction predictors accounted for 100% discrimination among dietary treatment by the model: *Keap1*.*UBE2H* and *Htr2cl1*.*Slc6a4a* (Figure 17b and Table 13).

Table 12 Results of the discriminant analysis performed for tested diets. Two significant roots were obtained; the number of significant predictors retained in the final model is also indicated

Eigen value	Canonical R	Wilk's Lambda	Chi-square	Df	p-value	Significant predictors [a]
7.62	0.94	0.05	37.30	4.00	<0.0001	1
1.29	0.75	0.44	10.40	1.00	0.0013	1

[a] Number of significant predictors ($p<0.05$) in each model

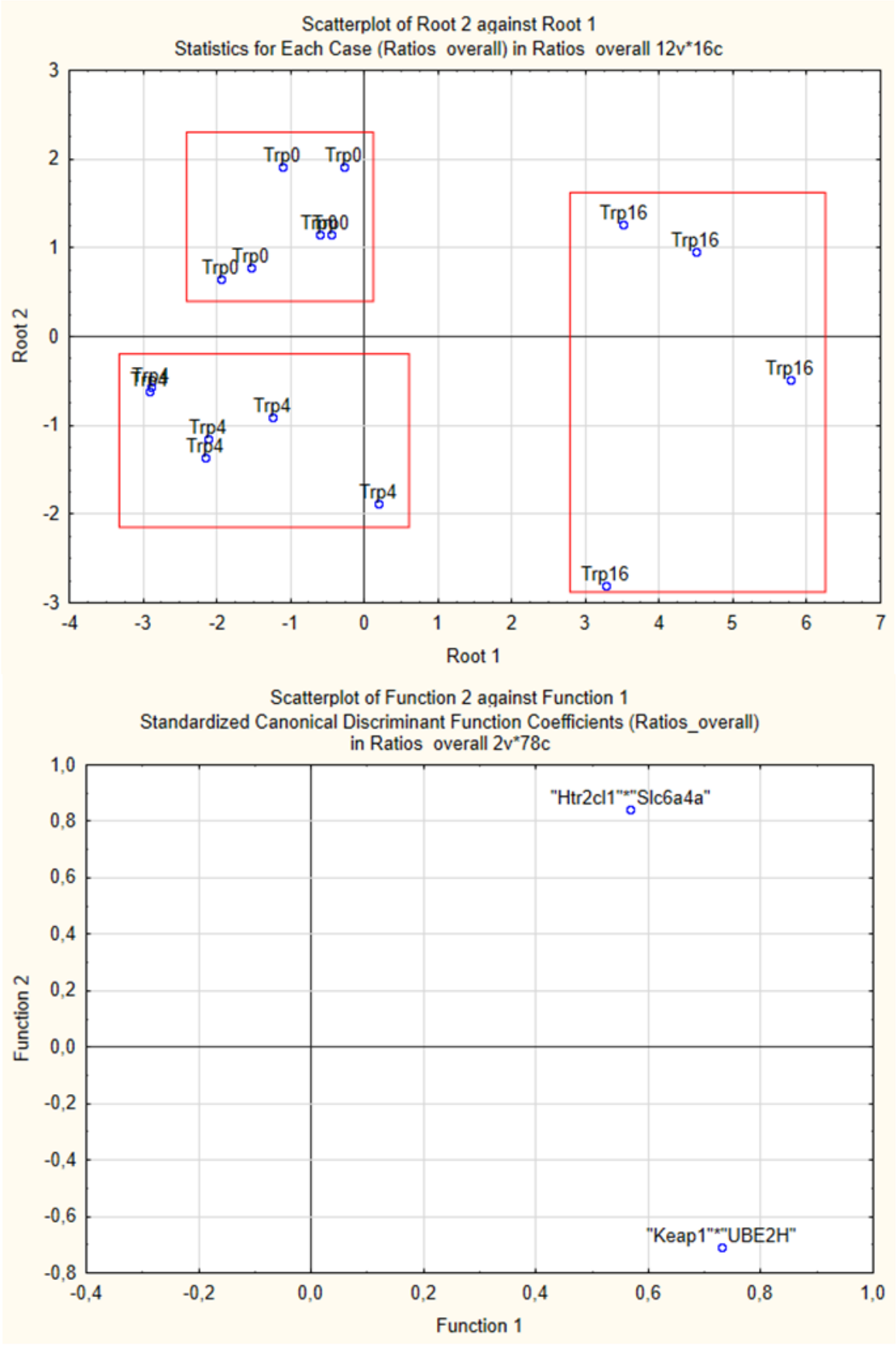


Figure 17 Scatterplot of both functions a) discrimination of dietary treatment; b) discrimination of predictors.

Table 13 Classification of functions obtained for the dietary treatment evaluated.

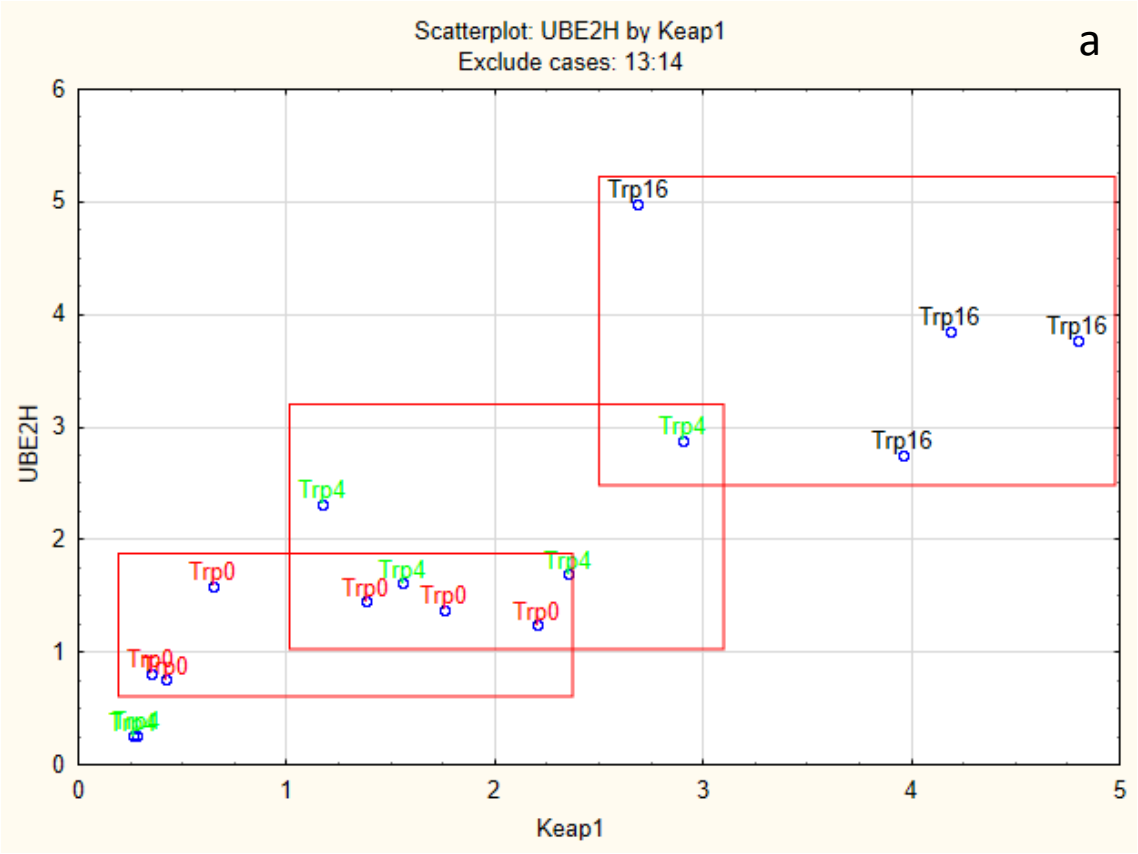
	Trp0 p=,3750	Trp4 p=,3750	Trp16 p=,2500
Intercept	-5,18	-1,75	-28,29
Htr2cl1.Slc6a4a	2,60	0,38	4,12
Keap1.UBE2H	0,03	0,43	1,97

Analysis of homogenous subsets produced by Tukey HSD test showed how each predictor discriminated dietary treatments (Table 14). For the *Keap1.UBE2H* predictor, expression levels of Keap1 generally increase with the expression of UBE2H (Figure 18a, Table 13). Moreover, fish fed the Trp0 and Trp4 diets showed lower expression of these genes than Trp16.

For the *Htr2cl1.Slc6a4a* moderate levels of expression were detected for fish fed Trp0 diet. (Figure 18b, Table 13). Fish fed the Trp16 diet exhibited moderate to high levels of expression of Htr2cl1 and Slc6a4a. Comparatively, animals fed Trp4 exhibited low levels of Htr2cl1 expression and moderate levels of Slc6a4a

Table 14 Homogeneous subsets obtained for each predictor retained in the final discriminant model, thought the Tukey HSD test

Predictor	Diet		
	Trp0	Trp4	Trp16
Keap1.UBE2H	1.47^{a}	2.96^{a}	14.60^{b}
Htr2cl1.Slc6a4a	3.22^{b}	0.68^{a}	6.05^{c}



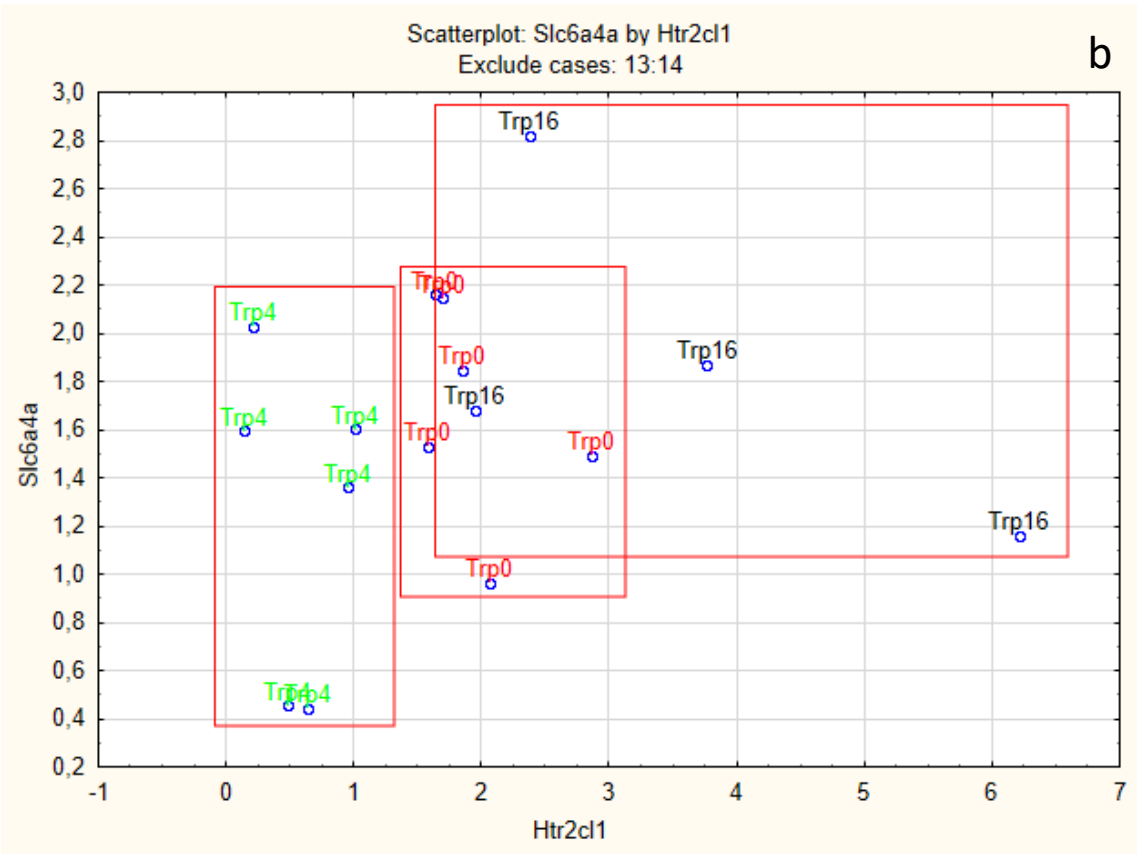


Figure 18 Scatterplot a) Slc6a4a by Htr2cl1, b) UBE2H by Keap1

Discussion

Amino acids, as well as protein, are needed to promote optimum fish growth, as growth is dependent on protein synthesis and deposition (Tang et al 2013). Essential amino acids (EAA), such as Trp must be supplied through diets, as they cannot be synthesized by the organism (Pianesso et al., 2015). A deficiency in an EAA may affect final weight and impair feed efficiency. Hoseini et al. (2017) reviewed Trp deficiency signs noticed in teleosts and reported skeletal deformities and implications in growth performance. Moreover, Trp requirements increase under stressful conditions such as those associated to intensive rearing. In the present study, no changes were observed on growth performance or feed utilization in response to dietary levels of Trp; however quadratic responses for increasing dietary Trp levels were detected for whole body gross energy content as well as for energy and nitrogen retention, with a peak in fish fed diets with intermediate levels of Trp (Trp8 and Trp4, respectively).

Requirements in Trp vary amongst fish species between 0.3-1.3% of dietary protein levels (reviewed in Hoseini et al., 2017), although, Trp requirements for zebrafish have yet to be established. Levels of Trp in the diet above the optimum level may depress growth performance (Murthy and Varghese, 1997; Ahmed and Khan, 2005; Ahmed, 2012, Pianesso et al., 2015; Zaminhan et al., 2018). For meagre, a total dietary Trp level of 2.49% (DM) led to negative effects on growth, despite not affecting feed intake (unpublished data). Papoutsoglou et al. (2005a, b) have shown that supplementation of Trp decreased growth performance but increased food consumption in rainbow trout and European sea bass. Hseu et al. (2003) reported a limitation in food intake upon supplementation of Trp, which the authors attributed to an increase in serotonergic activity. Indeed, studies have reported that 5-HT can be linked to an increase in satiety in rats (Blundell, 1986; Coşkun et al, 2006; Voigt and Fink, 2015; Shibui et al., 2017). Possible deleterious effects of Trp may be attributed to a disproportional intake and can be explained through antagonistic actions against other amino acids (Papoutsoglou et al., 2005b), impairment in absorption or toxic effect. Also, poor growth of fish fed diets with high Trp levels may be linked to the use of the amino acid for energetic purposes, because excess Trp is probably deaminated and excreted as ammonia (Walton, 1985).

As aforementioned, fish growth is dependent on protein synthesis. Amino acid supplementation can influence expression of genes related to protein turnover and growth regulation (Rolland et al., 2015). Studies report a correlation between growth hormone/insulin growth like-factor hormone (GH/IGF) system and growth, especially between IGF1 and growth (Beckman, 2011, Rolland et al., 2015). Metabolic active tissues, such as the liver, have higher protein synthesis, mostly because the liver plays an important role in amino acid metabolism as

well as synthesis and exportation of proteins (Fraser and Rogers, 2007). Indeed, liver is the main site of expression of IGF1 (Rolland et al., 2015). Gaylord et al. (2005) studied the Trp requirements for hybrid striped bass juveniles, and reported that deficiency in Trp can lead to a reduction in plasma IGF1 levels, and that Trp supplementation can increase IGF1 levels. Present results show that Trp supplementation did not affect significantly liver expression of IGF1, despite a slight increase with diets Trp4 and Trp16. Gaylord et al. (2005) also showed in the same report that increasing levels of Trp resulted in an increase in growth, contrary to the deficient diets which decreased growth performance. Also, Zhao et al., (2019) showed that supplementation of Trp increased liver expression of IGF1 and IGF2 until 4.2g Trp kg^{-1}diet. However, in the present study, Trp levels tested did not affect growth performance despite the referred slight increase observed in IGF1 gene expression.

The GH/IGF system has been reported as a regulator of protein synthesis through the TOR pathway. TOR plays an important role in protein synthesis, as it is activated by an amino acid pool (Tang et al., 2013). Tang et al. (2013) studied the effects of Trp supplementation in TOR expression in several tissues (hepatopancreas, proximal, mid and distal intestine and muscle) of common carp, and reported a decrease in TOR expression with the increase of dietary Trp. Similarly, Wen et al. (2014) evaluated the TOR expression in the intestine of grass carp, showing that increasing levels of Trp down-regulated TOR expression levels in the proximal, mid and distal intestine. Contrarily, in present results, a linear tendency was encountered for TOR expression levels, showing that with increasing levels of dietary Trp a higher TOR expression levels on the liver were encountered. This could be attributed to the important role in protein synthesis played by the liver, as previously mentioned. Similarly, for hybrid catfish, Zhao et al. (2019) demonstrated that supplementation of Trp increased TOR expression in the liver up to 4.2g Trp kg^{-1}diet. Moreover, Jian et al (2015) showed that increasing levels of Trp up-regulated TOR expression in the gills of grass carp.

Protein degradation is important in the assessment of protein turnover. Degradation of proteins is achieved through different pathways all controlled by IGFs. The Ubiquitin Proteosomase pathway (UPP) is one of these degradation pathways, degrading intracellular short-lived or abnormal proteins Protein degradation is important for several reasons, as it is firstly a way to control protein turnover and eliminate non-functional or denatured proteins (Fraser and Rogers, 2007; Rolland et al., 2015). The UPP involves the use of specific enzymes, such as ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3) (Kobayashi et al., 2004, 2006; Meyer-Schwesinger, 2019). Correlation map showed that IGF1 and UBE2H are highly correlated, which sustains that IGF controls this degradation pathway. In this study the supplementation of Trp did not affect expression levels of UBE2H. When protein turnover is balanced, protein synthesis and protein degradation are equal (Fraser and Rogers,

2007). Present results indicate a similar expression of UBE2H and TOR genes, probably meaning a balance between protein synthesis and protein degradation. Thus, the balance in protein turnover would be in accordance with growth results, as supplementation of Trp did not influence growth performance.

As aforementioned, no reports were found evaluating Trp requirements for zebrafish. Therefore, dietary Trp levels used in this trial for zebrafish may have been, as the non-supplemented diet (Trp0) did not impair growth performance, nor deficiency signs were observed. Since information on Trp requirements of zebrafish is not available, we used the Trp requirements for common carp (also a cyprinid) - 0.3% DM (Dabrowski, 1981) – as a basis to set the tested range of Trp levels for this trial. Indeed, it is a common practice that other cyprinid requirements are used for zebrafish, as their nutritional requirements have yet to be fully established and there isn't a standard (Lawrence, 2007; Siccardi et al., 2009; Kaushik et al., 2011; Kent and Varga, 2012; Penglase et al., 2012; Watts, 2012; Fernandes, 2016). Standard diets are a promising way to minimize unintended nutritional effects (Bolker, 2012), as variations or lack in specific nutrients can lead to several alterations in the organism (Fowler et al., 2019). The lack of a standard diet can lead to variability in research results (Siccardi et al., 2009; Lawrence, 2011; Watts et al., 2012) and have a strong impact on the reproducibility of trial, thus limiting the success of zebrafish as a research model such as zebrafish (Watts et al., 2012). Indeed, Fowler et al. (2019) showed that different diets even with the same feeding protocols lead to variation in growth after sixteen weeks in zebrafish, supporting that different diets assure different outcomes and emphasize the need for a standard diet. Given the lack of studies regarding nutritional requirements for zebrafish, further research needs to be conducted in order to achieve a standard diet to assure maximum growth performance and studies reproducibility.

Trp is the precursor of 5-HT, and it's the only limiting factor to the conversion process. Trp conversion into 5-HT occurs in the brain and gastrointestinal tract. Synthesis of 5-HT is commonly mentioned as a dose-response mechanism dependent on Trp availability, which is conditioned by Trp transportation to the brain through the same transporter as other LNAA (Le Floc'h et al, 2011; Hoseini et al., 2017). The serotonin pathway is not the most relevant pathway of Trp degradation, as most Trp is degraded through the kynurenine pathway which mainly occurs in brain and liver (Wu, 2013). In order to form 5-HT, Trp suffers the action of Tryptophan Hydroxylase (TPH) and an aromatic amino acid decarboxylase with the presence of vitamin B_6 to form 5-HT (Hoseini et al., 2017). TPH is the rate-limiting enzyme to serotonin synthesis and it is not saturated by its subtract nor inhibited by 5-HT, meaning it will continuously transform Trp into 5-HT (Lepage et al., 2003; Winberg and Thörnqvist, 2016; Hoseini et al., 2017). In zebrafish brain, four isoforms of TPH have been found, TPH1a, TPH1b, TPH2, and TPH3 (Höglund et al., 2019). TPH2 was found in other organs aside from the brain, suggesting that TPH can be found

in peripheral organs aside from the brain also in fish (Rahman et al., 2011). In the present study, three isoforms were studied TPH1a, TPH1b, and TPH2, in the case of TPH2, included the variants, TPH2a and TPH2b. Peripheral 5-HT synthesis is also present in other organs, such as liver, stomach and gills epithelium (Hoseini et al., 2017). According to present results, TPH1a decreased linearly with the increase in dietary Trp; and TPH1b seems to compensate for this decrease. Since, the serotonin pathway is not the main Trp degradation pathway in liver, this can explain the lower values of expression. Indeed, serotonin pathway mainly occurs in the brain and gastrointestinal tract, and as aforementioned only peripheral synthesis occurs in other organs such as the liver (Hoseini et al., 2017).

Studies in both mammals and teleosts suggest that increasing dietary levels of Trp leads to an increase in its uptake to the brain, therefore, increasing 5-HT levels (Machado et al., 2019). Reports have also shown that Trp supplementation produces similar effects to the ones reported for 5-HT administration (Hoseini et al., 2017). Present results show that whole brain serotonin levels did increase with the increase of dietary Trp with a linear tendency, being in accordance with the consensus that increasing Trp availability increases serotonin production (Höglund et al., 2007). In mammals, 5-HT receptors are divided into three families, 5-HT_1, 5-HT_2 and 5-HT_3. However, in fish due to genome duplication, genes have subtypes for instance Htr1aa (5-HT_{1AA}) and Htr1ab (5-HT_{1AB}), with divergent expression (Backström and Winberg, 2017). Trp supplementation is supposed to enhance serotonergic synaptic transmission, with changes to serotonergic receptors expression and sensitivity. Increase in 5-HT during a long-term period due to stress, can delay up-regulation of 5-HT neurotransmission (Lepage et al., 2005b; Höglund et al., 2017). In the present work, there was decrease in Htr2cl expression level in fish fed diet Trp4, and in Htr1aa expression level in fish fed diets Trp4 and Trp16. As these receptors are more common in the brain, lower levels of expression are expected in peripheral organs; moreover, since 99% of Trp is used in the kynurenine pathway, another possibility for this difference can be a higher rate of degradation through that pathway.

Serotonin transporter is responsible for serotonin reuptake, affecting post-synaptic binding of 5-HT. In animal models, depletion of brain 5-HT was reported to decline expression of 5-HT transporters (Praschak-Rieder et al., 2005). Similarly to what happens for the serotonin receptors, zebrafish possess two paralogs for the serotonin reuptake transporter: Slc6a4a and Slc6a4b (Backström and Winberg, 2017), with divergent expression. In the present work, Trp supplementation did not affect Slc6a4a expression level.

Usually, serotonergic activity is measured through the ratio 5-HIAA/5-HT, which indicates the conversion of 5-HT into its main metabolite, 5-HIAA (Hoseini et al., 2017). An increase in the 5-HIAA/5-HT ratio may be coupled with an increase in monoamine oxidase (MAO) activity,

as MAO mediates this conversion. Indeed, after 5-HT is released from synaptic vesicles into the synaptic cleft and its binding to receptors, a reuptake mechanism removes 5-HT from the cleft back to the presynaptic neuron, where MAO metabolizes it into 5-HIAA. A study on rainbow trout (Lepage, 2002, 2003, 2005b) reports that the 5-HIAA/5-HT ratio enhances with increasing Trp. In this study, neither MAO activity nor expression, as well as 5-HIAA levels, were evaluated; therefore the 5-HIAA/5-HT ratio was not calculated.

Trp is mostly degraded by action of indoleamine 2,3-dioxygenase (IDO) or tryptophan 2,3-dioxygenase (TDO) forming *N*-formylkynurenine, which is then converted in Kynurenine. Although the mechanism is present in teleosts, fish are unable to produce substantial amounts of niacin, meaning this pathway serves mainly as excretion of excess Trp (Hosseini et al., 2017). IDO and TDO vary in several aspects including tissues where they are most present, which is liver in the case of TDO (Le Floc'h et al., 2011, Hoseini et al., 2017). This pathway degrades nearly 99% of ingested Trp, leaving less than 1% for serotonin synthesis. However, the kynurenine-niacin pathway, its enzymes or sub-products were not evaluated in this report.

Acetylcholine (ACh) is an important neurotransmitter present in the central nervous system (Venkataraman et al, 2008), and its action is dependent on the presence of acetylcholinesterase (AChE), an enzyme responsible for degradating ACh. AChE is frequently used as a biomarker of toxicity. Studies show that when AChE is inhibited, ACh can accumulate and over stimulate cells, leading to hyperactivity, and even death of the organism (Olney et al., 1986; Rodrigues et al., 2013). According to some studies, zebrafish only possesses the AChE gene (Bertrand et al, 2000; Richetti et al., 2011; Koening et al., 2016) meaning degradation of ACh is performed only by action of AChE.

Inhibition of AChE can affect movement by affecting the neuromuscular function. Actually, the activity of this enzyme is highly important for functions such as locomotion, evasion and orientation (Golombieski et al., 2010). For instance, the inhibition of AChE activity by organophosphate quinalphos drastically change the swimming behavior of common carp (Chebbi and David, 2009). In the present study, AChE activity was two times higher in the muscle when compared to the brain. Golombieski et al. (2010) also reported a higher activity in the muscle than in the brain, which was also a two-fold higher AChE in the muscle than in the brain of bighead carp juveniles.

Tejpal et al. (2009) evaluated the effect of dietary Trp levels on crowding stress in mrigal carp, found that brain AChE activity increased with increasing levels of Trp. Present results show that although Trp tested levels did not affect AChE activity in the muscle, a quadratic response was found in the brain, where a Trp supplementation higher than that of the Trp4 diet induced a decrease in AChE activity. Since, ACh receptors may be sensitive to other neurotransmitters, such

as serotonin (Balasubramanian and Bhanumathy, 1993; Toledo-Ibarra et al., 2013), an increasing availability of brain serotonin, can limit the binding of ACh to its receptor. Indeed, reports have suggested an association between the cholinergic system and other neurotransmitters in zebrafish (Schmidel et al., 2014).

Dietary Trp supplementation has been pointed out as a possible nutritional strategy to mitigate stress, due to the role of this amino acid in serotonin and melatonin synthesis (Lepage et al., 2005a, Martins et al., 2013). These products of tryptophan degradation have important roles in antioxidant responses. Moreover, it was suggested that tryptophan itself can have antioxidant action, as it modulates antioxidant enzyme activity (Hoseini et al., 2017). Some studies report that metabolites from Trp degradation can act as ROS scavengers and modulate antioxidant enzymes (Hoseini et al., 2017). Nrf2 gene regulates cellular antioxidant response. At a resting state, Nrf2 and Keap1 combine in the cytoplasm and Keap1 promotes proteosomal degradation of Nrf2, after stimuli, Keap1 and Nrf2 uncouple, and Nrf2 can be transferred to the nucleus, Trp can activate the expression of antioxidant enzymes, through the inhibition of Nrf2 degradation, allowing Nrf2 translocation into the nucleus. Keap1 acts as a repressor of Nrf2 (Zheng et al., 2016). Wen et al., (2014) showed that Trp supplementation increased Nrf2 gene expression as it decreased Keap1 gene expression, and thus Nrf2 gene expression was negatively correlated to Keap1expression in the intestine. The same study reports, that anti-oxidant enzymes mRNA expression levels increased with Trp supplementation. The majority of Trp is degradated through the kynurenine pathway and this pathway produces a metabolite (3-hydroxyanthranilic acid) by TDO and kynurenase action. This metabolite can induce dissociation between Keap1 and Nrf2, promoting the translocation of Nrf2 to the nucleus and consequent expression (Xu et al., 2018). In rat liver, melatonin up-regulated Nrf2 expression (Jung et al., 2010), a similar effect to that observed in hybrid catfish intestine due to Trp dietary supplementation (Zhao et al., 2019). In hybrid catfish intestine, Nrf2 was up-regulated with the maximum of expression at 4.2gTrp kg^{-1} of diet, while Keap1 expression hit the lowest value for that same dietary treatment, highlighting the negative correlation in the expression of these two genes. Contrarily to these results, present study shows that supplementation of Trp induced the expression of Keap1 and inhibited Nrf2 expression. This could may indicate that the production of Keap1 is promoting proteosomal degradation of Nrf2, down-regulating its expression. A possible explanation is that tested Trp levels were not enough to suppress Keap1. Since, studies haven't focused on the effects of Trp on this pathway in teleosts, more research should be conducted in order to clarify these issues.

Multivariate analysis fully discriminated tested diets based on the expression levels of four of the twelve measured genes. These genes belong to the signaling pathway of serotonergic neurotransmission (serotonin receptor, Htr2cl1 and serotonin transporter, Slc6a4a), protein degradation from the UPP (UBE2H) and antioxidant mechanisms (Keap1). A high

discrimination was achieved as a result from accounting potential interactions or concomitant responses. The two by two interaction predictors provided further mechanistic knowledge on liver effects of Trp supplementation. On the one hand, these results sustain the relationship between serotonin receptor and transporter, recognized for the central nervous system. On the other hand, present results highlight a crosstalk between the UBE2H and Keap1 pathways. Indeed, according to literature, Keap1 targets Nrf2 for ubiquination and subsequent degradation by the proteosome; however this occurs associated with an ubiquitin ligase E3 complex, with at least a conjugating enzyme (Zhang and Hannick, 2003, Plafker et al., 2010). Keap1 is an adaptor for an ubiquitin ligase (Fuse and Kobayashi, 2017). Zebrafish has a similar Keap1-Nrf2 system to mammals, with this system being highly conserved (Fuse and Kobayashi, 2017). Present results, show that Keap1 and ubiquitin conjugating enzyme E2 (UBE2H) are highly correlated. Moreover, expression levels of Keap1 increased with increasing expression of UBE2H, showing a higher expression of these genes with Trp supplementation. However, further investigation should be conducted to fully understand the regulation of the Keap1-Nrf2 system. The expression levels of Keap1, UBE2H, Htr2cl1, and Slc6a4a appear to be a trimmed useful tool to assess dietary Trp supplementation.

Trp has been shown to modulate fish behavior (see review Hoseini et al, 2017) with special emphasis on decreasing aggressive behavior and stress mitigation. Serotonin is also associated with mitigation of stress and suppression of aggressive behavior in teleosts has been attributed to serotonergic system (Hoseini et al., 2017). As more 5-HT is released through an increasing serotonergic activity, an indirect mechanism activated translating into behavioral and neuroendocrine effects, which is related to cortisol release, by regulation of the corticotrophin-releasing factor (Höglund et al., 2007). Aside from aggression, cannibalism was also studied in pike perch and grouper, and, in both species, Trp supplementation in diets decreased cannibalism; however mechanistic components for this response have yet to be cleared (Höglund et al, 2019). Effects of Trp on feeding behavior have also been studied, with contradictory results as aforementioned.

Melatonin is a hormone responsible for several physiological responses aside from those controlled by the circadian rhythms (Hoseini et al., 2017). Moreover, melatonin is linked to locomotor activity (Gaildrat and Falcón, 2000). In *Dicentrarchus labrax* both melatonin and Trp administrated for 7 days reduced locomotor activity (Herrero et al., 2007). In zebrafish, administration of melatonin was shown to decrease locomotor activity and induced a lethargic state (sleep-like state) (Zhdanova et al., 2001). Tryptophan is an indirect precursor of melatonin synthesis, as melatonin is obtained through serotonin, Trp administration was shown to increase melatonin levels (Hoseini et al., 2017). Although melatonin levels were not evaluated in the present work, we assessed the effect of Trp supplementation on locomotor behavior of zebrafish..

Changes to standard behavior can indicate some sort of disturbance, such as stress, inadequate conditions, distress or disease (Conte, 2004). Decreasing locomotor activity can be associated to energy saving, reduction of exploration and diminished risk of predation (Kristen et al., 2018). Both higher and lower velocities can be associated with disturbances to the fish. Swimming patterns are highly sensible and ecological relevant, allowing the detection of disturbances (Janz, 2011). Interaction between behavior and amino acid supplementation has only been recently addressed. In our study, distinctive locomotor behaviors were detected depending on the dietary treatment. Fish fed the diet with the highest level of Trp (trp16) were associated to a behavior characterized by minimum velocity, meaning fish were more lethargic, corroborating previous findings referred above. In opposition, fish fed the Trp non-supplemented diet (Trp0) were related to a behavior characterized by maximum velocity, which may be linked to lower serotonin levels in these fish. Additionally, fish fed the diet with intermediate Trp supplementation (trp4) were associated to a moderate velocity, meaning a more uniform locomotion. In other words, fish fed a diet with an intermediate Trp level (Trp4) displayed an average locomotion that can be associated to a less stressed behavior, while those fish fed diets with extreme Trp levels (Trp0 and Trp16) were associated with more stressed behaviors.

Conclusions

Dietary tryptophan levels from 0.22 to 3.34%DM had no significant impact on zebrafish growth and feed intake. Nevertheless, a quadratic response to increasing dietary Trp levels was found for some indicators of feed utilization, such as nitrogen and energy retention. Some biochemical parameters were also affected by the amount of Trp in diet: brain serotonin levels increased linearly with increasing dietary Trp levels, and brain acetylcholinesterase activity showed a quadratic response to increasing dietary Trp levels. Also, the expression of some selected genes involved in the serotonin pathway, protein synthesis/degradation, antioxidant defense and Trp degradation were influenced by dietary Trp levels. TPH1a decreased linearly, Htr2cl1 had lower relative expression in diet Trp4 and Htr1aa in diets Trp4 and 16. TOR relative expression increased linearly as well as Keap1. Finally, distinctive locomotor behaviors were detected which can be associated to a more or less stressed condition depending on the dietary Trp level. Overall, diet Trp4 (0.91% DM) appears to be the most equilibrated diet for zebrafish.

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Appendix:

1) Spectophotometric Method for Determining Tryptophan in Diets according to DeVries et al. (1980)

Solutions required:

1. Phospate Buffer- Sodium phosphate dibasic (4.40g) and potassium phosphate monobasic (4.40g) dissolved into 1L of distilled water (pH 7.5)
2. Pronase solution- 60 mg Pronase into 15 mL of Phosphate Buffer
3. Sulfuric Acid (21.2N)- 25mL Concentrated Sulfuric acid added to 85mL of distilled water (flask swirled under cold tap water). Solution cooled to room temperature before use.
4. P-(Dimethylamino)benzaldehyde (DAB)- 0.94g DAB mixed with with 250 mL of sulfuric acid.
5. Sodium nitrite – 24mg of sodium nitrite is dissolved in 50mL of distilled water.

Procedure:

1. Take 25-30mg of sample into a flask
2. Add 0.5mL of Pronase solution
3. Place the flask on the ultrasonic bath until wetted
4. Add 1 drop of Toluene
5. Place samples in a 40±1°C bath for 24 hours
6. Cool the samples to room temperature
7. Add phosphate buffer to bring the volume to 5mL and vortex to mix
8. Prepare DAB solution
9. On clean flasks, add 4mL of DAB solution+1mL of the hydrolyzed and vortex to mix
10. Let it overnight on a dark place
11. Add 0.05mL of sodium nitrite
12. Mix using the vortex
13. Rest for 30 minutes to gain color
14. Filter using a syringe and a Nylon filter and place the filtrated in new flasks

15. Read absorbance at 590nm

Calculations:

Sample blank absorbance was subtracted from the sample absorbance where applicable

The micrograms of Trp present in the sample were determined using the following formula:

$$Trp(\%) = \frac{0.1T}{S}$$

Where:

T- Micrograms of Trp present; S- milligrams of sample; 0.1- Conversion Factor µg to mg