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Evaluation of protein requirements for juvenile growth and effect of dietary protein levels on post-juvenile growth and reproduction of zebrafish (*Danio rerio)*

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Abstract

Despite the increasing utilisation of zebrafish as research model and the investment in its large-scale production, zebrafish rearing conditions are still based on subjective experience and conditions found for other aquacultured species. Since standardization and optimization of rearing conditions are fundamental to ensure reproducibility and reliability of research outputs, it is indispensable to perform studies especially designed to define specific conditions for zebrafish rearing. This necessarily includes the evaluation of nutritional requirements of zebrafish and the subsequent formulation of a standard diet. In view of these considerations, the present work aiming at (1) assessing dietary protein requirement for juvenile growth of zebrafish, and (2) assessing the effect of dietary protein levels on post-juvenile growth and reproductive performance of zebrafish.

In a first experiment ten isoenergetic diets containing between 15% and 60% of protein were formulated in order to estimate the protein requirements for zebrafish juvenile. Dietary protein requirements were estimated at about 37% for maximum weight gain, 47% for maximum nitrogen retention, and 45% for maximum energy retention. A daily ration of 33.6 g kg^{-1} of a diet containing 37% of protein and a daily ration of 29.1 g kg^{-1} of a diet containing 47% of protein will promote respectively maximum weight gain and maximum nitrogen retention in zebrafish juvenile.

In a second experiment four isoenergetic diets containing between 30% and 60% of protein were formulated in order to evaluate the effect of dietary protein levels on growth and reproductive performance of zebrafish post-juveniles. Neither growth nor the reproductive performance of zebrafish post-juvenile was significantly influenced by tested dietary protein levels, suggesting that relatively low dietary protein levels can be used in zebrafish husbandry at this stage.

In our opinion, overall results should be viewed as a contribution towards the formulation of standard diets for zebrafish juvenile and post-juvenile stages, aiming the general standardization of zebrafish husbandry.

Keywords

Zebrafish, protein, juvenile phase, post-juvenile phase, growth, reproduction.

Resumo

Apesar da utilização crescente do peixe-zebra como modelo biológico e do investimento na sua produção em grande escala, as suas condições de cultivo são ainda baseadas na experiência subjetiva e nas condições estabelecidas para outras espécies de aquacultura. Uma vez que a padronização e otimização das condições de cultivo são fundamentais para assegurar a reprodutibilidade e a consistência dos resultados de investigação, torna-se indispensável realizar estudos especialmente concebidos para definir as condições de cultivo do peixe-zebra. Tal deverá incluir necessariamente a avaliação das necessidades nutricionais desta espécie e a subsequente formulação de uma dieta padrão. Tendo em vista estas considerações, o presente trabalho tem como objetivos (1) determinar as necessidades proteicas para o crescimento de juvenis de peixe-zebra, e (2) avaliar o feito de diferentes níveis proteicos da dieta no crescimento e desempenho reprodutivo de pós-juvenis.

Numa primeira experiência foram formuladas dez dietas isoenergéticas com teores proteicos entre 15% e 60% para estimar as necessidades proteicas dos juvenis. Essas necessidades foram estimadas em cerca de 37% para o ganho de peso máximo, 47% para a máxima retenção azotada e 45% para a máxima retenção energética. Uma ração diária de 33,6 g kg^{-1} de uma dieta com 37% de proteína e uma ração diária de 29,1 g kg^{-1} de uma dieta com 47% de proteína promoverá respetivamente um ganho de peso e retenção azotada máximos.

Numa segunda experiência foram formuladas quatro dietas isoenergéticas com teores proteicos entre 30% e 60% para avaliar o efeito do nível proteico das dietas no crescimento e desempenho reprodutivo de pós-juvenis de peixe-zebra. Nem o crescimento nem o desempenho reprodutivo foram significativamente influenciados pelos níveis proteicos testados, o que sugere que poderão ser utilizadas dietas com níveis relativamente baixos de proteína no cultivo do peixe-zebra nesta fase do ciclo de vida.

Na nossa opinião, o conjunto dos resultados obtidos deverá ser considerado como uma contribuição com vista à formulação de dietas padrão para as fases juvenil e pós-juvenil.

Palavras-chave

Peixe-zebra, proteína, fase juvenil, fase pós-juvenil, crescimento, reprodução.

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General introduction

Notes on the biology of zebrafish (*Danio rerio*)

The zebrafish *Danio rerio* is a teleost fish belonging to the Cyprinidae Family. It has a fusiform and laterally flattened body, with alternated dark and bright stripes throughout its extension (figure 1), reason by which is commonly called zebrafish (Spence *et al.*, 2008). It is a usual aquarium ornamental fish but is also extremely important as research model in a wide range of scientific areas.

Zebrafish is native from south Asia, in countries like Pakistan, Bangladesh, Nepal, India and Myanmar (Spence *et al.*, 2008). These regions have a pronounced monsoon climate, with well-established rainy and dry seasons, influencing water parameters and abundance of resources, like food availability (Lawrence, 2007; Spence *et al.*, 2008). Recently Arunachalam *et al.* (2013) referred that zebrafish is often found in low flow and sandy substrate habitats in subsidiary channels of rivers or streams, and even in adjacent areas of wetlands and rice fields, either in natural canals or in man-made canals and ponds.

Figure 1: Zebrafish specimen **(Dowling, 2012)**.

Zebrafish exhibit a limited growth, not exceeding 5 cm of length and show sexual dimorphism: females are generally larger than males and have a rounded shape body due to the presence of eggs in the oviducts while males possess a slender and

darker body. In the beginning of its development all individuals are initially females possessing gonads similar to ovaries (Spence *et al.*, 2008). At around 5 to 7 weeks after hatching (10 to 15 mm of total length, TL) males start to develop testicles, experiencing an intersexual phase, and by the third month (12 to 17 mm TL) the process of gonadal differentiation becomes complete. The gonadal development rate depends on the rearing conditions as well as genetic features (Maack & Segner, 2003; Spence *et al.,* 2008).

Zebrafish has a typical shoaling behaviour and in the natural habitat can constitute groups ranging from 5 to 20 individuals (Spence *et al.*, 2008). From the reproductive point of view, zebrafish is an asynchronous species, spawning in small groups. The time at which individuals reach sexual maturity seems to be more related with the size rather than with the age. Spence *et al.* (2008) affirm that wild and laboratory strains seem to reach sexual maturity at similar sizes despite divergent growth rates. During the reproduction season in nature, males are territorial and actively chase the females. These, in turn, seem to guide their reproductive choices through olfactory cues and food availability (references in Lawrence, 2007; Spence *et al.*, 2008). Females release the eggs to the substrate and these are fertilized by males' sperm. In nature hatching occurs between 4 to 7 days into swimming larvae. The adults don't have parental care (Breder and Rosen, 1996; cited by Lawrence, 2007). In controlled laboratory conditions, males and females kept together can spawn in a continuously and frequent but irregular periods of time (Spence *et al.,* 2008).

Since the work of Kimmel *et al.* (1995), there has been an effort to accurately define the different life stages of zebrafish and, most importantly, define them in the most simple and precise form. After hatching, the life cycle of zebrafish can be divided in three distinct stages, based on body standard length (SL) and days post-fertilization (*dpf*) at 28.5 ˚C, as suggested by Singleman & Holtzman (2014). The larval phase lasts approximately 6 weeks (3 to 12 mm SL) beginning at the 3rd *dpf*, and is characterized by exponential growth. Around the 45th *dpf* the individuals enter the juvenile phase (12-18 mm SL) during which they still experience an exponential growth (Spence *et al.,* 2008) and undergo morphological changes, such as the complete loss of larval fin fold and acquisition of scales (Parichy *et al.*, 2009). Finally, the adult/post-juvenile stage occurs at around the 3rd month (90 *dpf*; length above 18 mm SL), at which growth rates decrease and individuals reach sexual maturity and become able to reproduce (Singleman & Holtzman, 2014).

Zebrafish as a biological research model

In the last decade zebrafish has become a major research model in many fields of biology and biomedicine, including the study of human diseases. This utilisation of zebrafish as a biological model has been constantly growing since 1951 (figure 2), the year of the first scientific paper published using this vertebrate. From 1996 the use of this species in research has grown exponentially, resulting in the publication of 8370 scientific papers in 2012 (Kinth *et al.,* 2013).

Zebrafish owns a group of traits that makes it an excellent research model in a wide range of scientific areas.

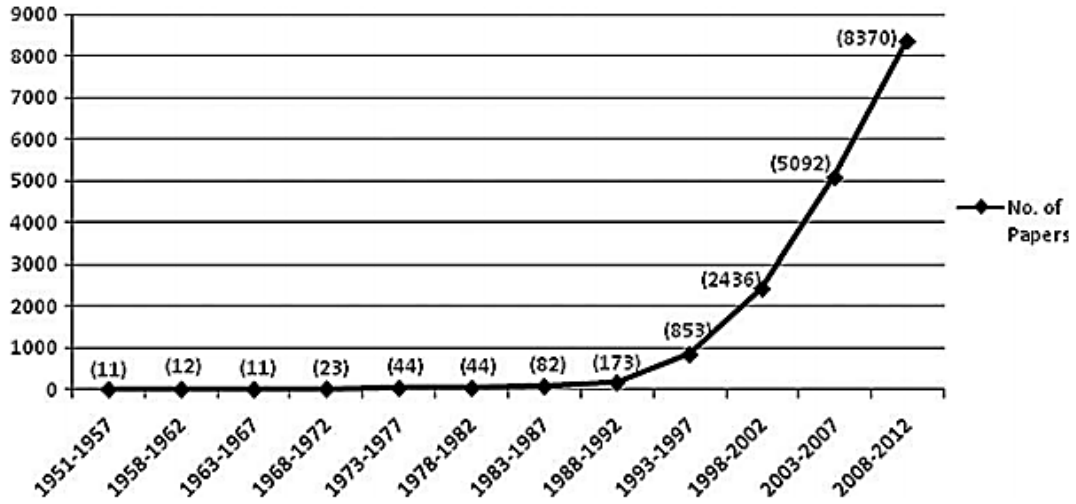

Figure 2: Number of scientific papers using zebrafish as a scientific research model from 1951 until 2012 (Kinth *et al.* , 2013).

Firstly, its small size allows keeping a large number of individuals in a relatively small space. This is a great advantage to perform experiments in multiple individuals at the same time, providing a high number of replicates and/or different experimental conditions.

The short generation time (about 3-4 months from egg to sexual maturity) (Maack & Segner, 2003) gives the possibility of a constant supply of progeny, and the opportunity of renewing stocks easily, performing experiments in different life stages and assessing outcomes rapidly.

Zebrafish and human genomes share a high similarity (Lieschke & Currie, 2007; Deo & MacRae, 2011). Scientific areas such as cardiology, obesity and immunology,

among many others, can benefit from this trait, allowing the progress of fundamental knowledge in human physiology and diseases.

Under controlled culture conditions zebrafish females can spawn on a daily basis, providing a continuous source of eggs that hatch between 2 up to 3 days. According to Dahm & Geisler (2006) a single female can spawn around 200 eggs in a week and has a life expectancy oscillating from 2 to 4 years, which means that one female can produce from 15.000 to 35.000 eggs during its entire life time. This feature can be particularly important in genetic studies aiming at identifying new genes and their functions in vertebrates (Dahm & Geisler, 2006), and makes possible to perform genetic studies at a big scale basing the results in phenotype expression (Deo & MacRae, 2011).

The external fertilization makes the handling of live embryos possible and allows the monitoring of their different developmental stages (Kimmel *et al.*, 1995). During a large part of its early life individuals exhibit optical transparency that makes possible to perform experiments whose results need to be readily seen. This characteristic is of major importance and distinctive from others vertebrate models, because allows the direct visualisation of internal organs and the use of fluorescent markers that target exact cellular and physiologic responses (Deo & MacRae, 2011).

Finally, zebrafish is an organism that can be easily reared and maintained at the laboratory. In fact, it has been cultivated under a wide variety of rearing protocols and conditions all over the world and still has successful reproduction and growth rates. This allows keeping stocks of thousands of individuals without much labour and at relatively low cost.

Current status of zebrafish feeding and nutrition

Observations made in the natural habitat indicate that zebrafish is an omnivorous and euryphagous species, preferentially eating zooplankton and dipteran larvae. However, zebrafish diet in the wild can include a large variety of items, such as phytoplankton, remnants of vascular plants, arachnids, invertebrate eggs and detritus (Lawrence, 2007; Spence *et al.*, 2008; Watts *et al.*, 2012). Adults also prey eggs and larvae of their own species (Spence *et al.*, 2008). The usual practice in zebrafish

facilities is feeding juveniles and adults with commercial dry feeds, typically food flakes used for aquarium ornamental fish, sometimes with a supplement of live brine shrimp *Artemia*. Ration size and feeding frequency are variable among zebrafish laboratories, but can have a significant influence on growth and reproductive performance (Lawrence *et al.*, 2012). Larval stages are usually fed on live food, and the conventional feeding protocol includes cultured paramecia during the first days after hatching followed by *Artemia* nauplii up to the end of the larval period (3-4 weeks after hatching) (Westerfield, 1995). However, this feeding protocol can be simplified, since it was demonstrated that paramecia are not essential and zebrafish larvae can be reared only on *Artemia* nauplii with higher survival and growth rates (Carvalho *et al.*, 2006). Moreover, the replacement of paramecia by rotifers during the first few days of feeding can also improve survival and growth (Best *et al.*, 2010). More recently, it was found that zebrafish can even be reared exclusively on a formulated diet right from mouth opening with survival and growth rates comparable to those obtained with *Artemia* (Kaushik *et al.*, 2011).

Studies on zebrafish nutrition are scarce and zebrafish nutritional requirements are practically unknown. A number of studies have focused on fatty acids, enlightening fatty acid metabolism in zebrafish (Tocher *et al.*, 2001) and reporting the importance of dietary supplementation with highly unsaturated fatty acids to improve zebrafish growth (Meinelt *et al.*, 2000) and reproductive performance (Meinelt *et al.*,1999; Jaya-Ram *et al.*, 2008). In a recent study Smith *et al.* (2013) tested several dietary protein sources in zebrafish juveniles and found significant differences in growth and body composition. The remaining studies have been related to mineral and vitamin nutrition. Rowe & Eckhert (1999) demonstrated the essentiality of boron for zebrafish normal embryogenesis and Hawkyard *et al.* (2011) suggested health benefits to zebrafish larvae resulting from dietary enrichment with iodine. Alsop *et al.* (2008) revealed the fundamental role of retinoids in zebrafish reproduction. Finally, Yossa *et al.* (2014a, 2014b) assessed biotin requirements for zebrafish juvenile growth, and showed the importance of dietary biotin in zebrafish reproduction.

Since main basic nutritional requirements (such as protein, amino acids and lipids) of zebrafish have yet to be determined, it has been suggested that known requirements of other cyprinids can be an acceptable approach of zebrafish requirements (Lawrence, 2007; Watts *et al.*, 2012). However, as this approach does

not take into account possible species specificities, nutritional studies expressly delineated for zebrafish are imperative (Lawrence, 2007; Watts *et al*., 2012).

Rationale and aims of the present study

Despite the increasing utilisation of zebrafish as research model and the investment in its large-scale production, zebrafish rearing conditions are still based on subjective experience and conditions found for other aquacultured species, and therefore quite variable among laboratories. Since standardization and optimization of rearing conditions are fundamental to ensure reproducibility and reliability of research outputs, it is indispensable to perform studies especially designed to define specific conditions for zebrafish rearing (Lawrence, 2007; Reed & Jennings, 2010; Lawrence, 2011; Kent & Varga, 2012; Lawrence & Mason, 2012). Considering that zebrafish has been fed on commercial diets of undefined nutritional composition, studies must necessarily include the evaluation of nutritional requirements of zebrafish and the subsequent formulation of a standard diet (Penglase *et al.*, 2012). A standard diet with adequate nutritional composition will promote optimal growth and physiological status, minimizing the contribution of unintended nutritional effects to research outputs and allowing more consistent comparisons among experiments.

In view of the above considerations, and taken into account that (a) dietary protein requirements of zebrafish are unknown, (b) protein is of primary importance for their structural and functional roles, and represents the major nutrient in fish diets in terms of both relative amount and price, and (c) protein requirements can change along fish ontogeny, the present work is divided in two main parts, aiming at:

1) Assessing dietary protein requirement for juvenile growth of zebrafish;
2) Assessing the effect of dietary protein levels on post-juvenile growth and reproductive performance of zebrafish.

1. Evaluation of protein requirements for juvenile growth in zebrafish

1.1. Introduction

Somatic growth is essentially protein deposition in muscles, which is the net result between catabolic and anabolic pathways of protein metabolism (Valente *et al.*, 2013). Thus, protein provided in the diet has to be sufficient to assure both protein to deposit as to be used in the turnover. In general, fish have higher protein requirements than other vertebrates (Wilson, 2002). This makes protein the main nutrient in terms of relative amount in fish diets, with consequent impacts in the costs of feeds due the high price of protein sources (Islam & Tanaka, 2004). Furthermore, additional attention must be paid to excess of dietary protein relative to fish needs, since this will increase ammonia excretion and its subsequent accumulation in water and ammonia is highly toxic to fish at very low concentrations. Having in mind all these considerations, the evaluation of dietary protein requirements is of primary importance towards the formulation of a specific diet for a given fish species. Therefore, this first experiment was designed to evaluate protein requirements for growth of zebrafish at the juvenile stage, which is characterized by high growth rates.

1.2. Materials and methods

1.2.1. Experimental recirculating water system

Experiments were performed in a recirculation water system (Figure 3) similar to that described by Charlon & Bergot (1984). Experimental groups of fish were maintained inside experimental units consisting of 10-L plastic tanks. These tanks were siphoned daily to eliminate faeces and replaced by clean tanks every two weeks, for which fish were transferred. Water temperature was kept at 28 ± 1 ˚C and photoperiod set at 14 hours light/10 hours obscurity. Nitrites remained at 0.05 mg/L and ammonia below 0.01 mg/L.

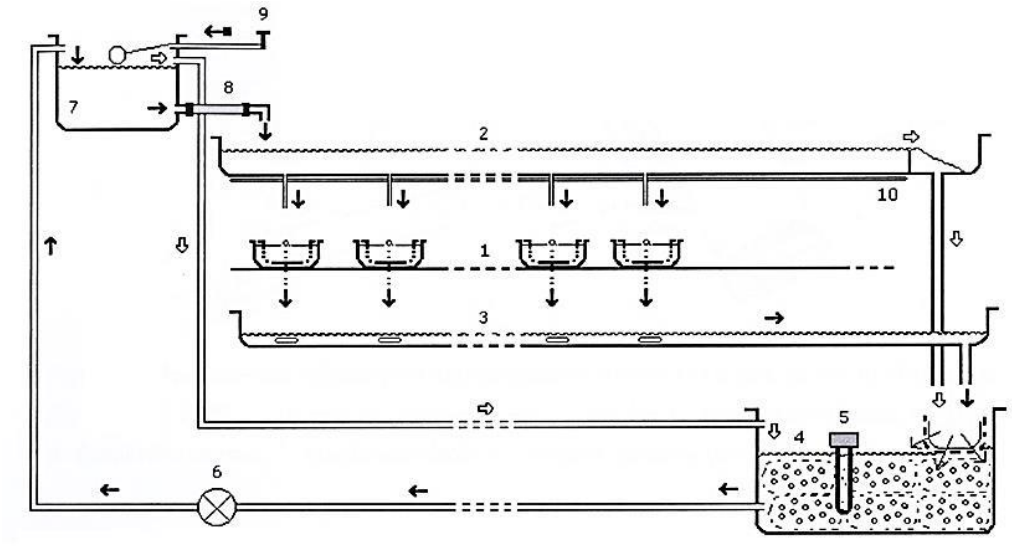

Figure 3: Scheme of the experimental recirculation water system. 1 – Experimental units; 2 – Water supply gutter; 3 – Water collecting gutter; 4 – Lower water reservoir with a biological filter; 5 – Heating water device; 6 – Water pump; 7 – Upper water reservoir with a security device; 8 – UV lamp; 9 – Water entrance into the security device; 10 – Fluorescent lamps.

1.2.2. Origin of experimental fish

Wild-type zebrafish newly-hatched larvae were obtained from the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR – University of Porto) and reared in the recirculating water system described above, according to the procedure reported by Charlon & Bergot (1984). After yolk resorption, larvae were fed rotifers in the first three days, *Artemia* nauplii from the fourth day up to 30 *dpf*, and the commercial flake feed *TetraMin* (47% protein, 10% lipids, 3% fibre, 11% ash, 6% moisture; package information) from 30 *dpf* until 54 *dpf*. Food was provided twice daily and seven days a week during the entire rearing period. At 54 *dpf*, already at the juvenile phase, a group of fish was used in this first experiment. Remaining fish were held under culture, fed the same commercial feed *TetraMin*, until reach sexual maturity and being used in the second experiment.

1.2.3. Experimental diets and experimental procedure

Ten isoenergetic diets were formulated to contain ten different protein levels ranging from 15% to 60% of dry matter. Diets formulation and respective proximate chemical composition are shown in Table 1.

Diets were randomly assigned to duplicated groups of 20 juvenile zebrafish, aged 54 *dpf*, with mean body weight of about 53.6 mg, and mean fork length of 17.8 mm at the start of the experiment. Fish were fed to apparent satiation twice daily, six days a week, throughout the experimental period of 59 days. To avoid any bias during feeding, each food container was coded with a number, instead of being identified with the respective diet, so it was not possible to know which diet was assigned to which tank. Aiming at adjusting food particle size to mouth opening, food particle size was 400-600 µm from 54 *dpf* up to 67 *dpf*, 400-600 µm and 600-1000 µm from 68 *dpf* up to 83 *dpf*, and 600-1000 µm from 84 *dpf* up to 113 *dpf*. One additional group was kept unfed along the experiment.

At the start of the experiment and every two weeks, fish in each tank were bulk weighed after 24 hours fasting, as well as the respective food containers in order to calculate food consumption. Fish weighing was taken with fish placed inside a beaker with water from the system. At the start of the experiment a sample of one hundred fish from the initial stock were euthanized with phenoxiethanol (0.3 mL/L) and frozen for later analysis of body composition. Another sample of ten fish from the initial stock was euthanized and photographed with a digital camera coupled to a stereomicroscope. Digital images were used to measure fish fork length with the software *ImageJ* (available in *http://imagej.nih.gov/ij/index.html*). At the end of the experiment all fish in each tank were euthanized with phenoxiethanol (0.3 mL/L), weighed as described above, measured from the snout until the caudal fin fork (fork length) as shown in Figure 4, and frozen for later analysis of body composition.

Table 1: Ingredients and proximate nutritional composition of the experimental diets.

	Dietary Protein Levels (%)									
Ingredients (g / kg diet in dry matter)	**15 P**	**20 P**	**25 P**	**30 P**	**35 P**	**40 P**	**45 P**	**50 P**	**55 P**	**60 P**
Fish meal [1]	176.4	246.5	316.5	386.6	456.6	526.7	596.8	666.8	736.9	807
CPSP [2]	30	30	30	30	30	30	30	30	30	30
Cod liver oil	77.7	71.1	64.6	58.1	51.6	45	38.5	32	25.5	18.9
Dicalcium phosphate	77.3	68.7	60.2	51.6	43	34.5	25.9	17.3	8.8	0.21
Mineral premix [3]	10	10	10	10	10	10	10	10	10	10
Vitamins premix [4]	5	5	5	5	5	5	5	5	5	5
Choline chloride (50%)	5	5	5	5	5	5	5	5	5	5
Binder [5]	10	10	10	10	10	10	10	10	10	10
Pregelatinized maize starch[6]	608.7	553.7	498.7	443.8	388.8	333.8	278.8	223.9	168.9	113.9
Proximate chemical composition (% dry weight)										
Crude protein	15.5	19	24.8	30	35	40.9	45.7	50	56.3	61.6
Ash	10.9	11.2	12.1	12.9	13.2	14.2	14.6	15.4	15.8	16.5
Crude lipid	7.8	8.0	7.7	7.4	8.0	8.1	8.3	8.8	9.4	9.5
Gross energy (kJ g^{-1})	18.36	18.26	17.96	18.33	18.62	18.20	18.71	19.17	18.89	18.91

[1] Pesquera Centinela, Steam Dried LT, Chile (CP: 71.4%; CL 9.3%). Sorgal, S.A. Ovar, Portugal
[2] Soluble fish protein concentrate, Sopropêche, France (CP: 80.4% DM; GL: 19.7% DM)
[3] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet).
[4] Vitamins (mg kg^{-1} diet): retinol, 18000 (IU kg^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.
[5] Aquacube. Agil, UK.
[6] C-Gel Instant – 12016, Cerestar, Mechelen, Belgium.

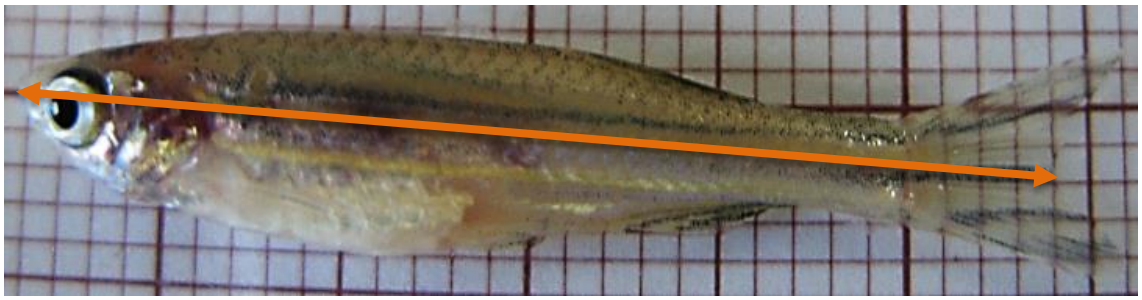

Figure 4: Individual for measurement of fork length at the end of the experiment. Photography by Helena Fernandes.

1.2.4. Chemical analysis

Diets proximate composition and fish body composition (in dried and homogenized samples) were analysed using the following standard methods: water content by drying samples in oven at 105 ˚C until constant weight; crude protein by the Kjeldahl' method, after acid digestion using a Kjeltec system; total lipids in a SoxTec System HT apparatus by petroleum ether extraction; ashes, after 16 hours incinerating in a muffle furnace at 450˚C; and energy through direct combustion of the samples in an adiabatic bomb calorimeter. Lipids were not determined in fish body composition due to low quantity of sample.

1.2.5. Statistical analysis

From data directly collected during the experiment (initial and final fish weight and fish length, food consumed, chemical composition of diets, and initial and final fish body chemical composition) were derived the following parameters indicative of fish growth performance, feed efficiency utilization, and nutrient intake and retention: Weight Gain (WG), Specific Growth Rate (SGR), Condition Factor Index, Length Variation Coefficient, Feed Efficiency (FE), Protein Efficiency Ratio (PER), Nitrogen Intake (NI), Nitrogen Retention (NR), Energy Intake (EI), and Energy Retention (ER).

Data were analysed by one-way analysis of variance (ANOVA) using the *IBM SPSS Statistics 22* software. If significant differences were detected ($p<0.05$) the Tukey multiple range test was used to discriminate means. All data were checked for

normal distribution and homogeneity of variance and when needed they were transformed.

The software *Statistica* was used for the estimation of protein and energy requirements. Different mathematical models were tested for the estimation of protein requirements but it was the four-parameter saturation kinetics model (4-SKM), developed initially by Morgan *et al.* (1975) and later by Mercer (1982) (references in Belal, 2005), that better fitted the obtained data. The equation is:

$$r = \frac{b\,(K0.5)^n + Rmax\ I^n}{K0.5 + I^n} \qquad \text{Where,}$$

r = physiologic response (weight gain or N retention); *I* = dietary protein level or nutrient intake; *b* = intersection in the r axis; R_{max} = theoretical maximum response; *n* = apparent kinetic order; $K_{0.5}$ = intake for half of (R_{max}+*b*) (Shearer, 2000; Belal, 2005). According to Belal (2005) this model efficiently describes the physiologic response by the animal if the experimental design includes a wide range of nutrient levels. Beyond that, it's a model that reaches a plateau with a predict asymptote each is a maximum response for a specific indicator (Shearer, 2000).

For the estimation of energy requirements was used a second-order polynomial equation (Belal, 2005):

$$Y = ax^2 + bx + c \qquad \text{Where,}$$

Y = physiologic response (ex: weight gain); *x* = amount of the nutrient; *a* and *b* = slopes of the curve; *c* = corresponds to the response at zero nutrient intake level.

1.3. Results

Growth performance and feed utilisation efficiency are summarized in Table 2. Increasing levels of dietary protein led to increasing weight gain although no significant differences were detected to protein levels above 25% ($p<0.05$). The feed intake was higher in fish fed with lower protein levels. The PER was higher for the lowest dietary

protein levels of 15 and 20% and decreased as dietary protein level increased. The feed efficiency increased along with the protein increment in diets, but for protein levels above 35% no significant differences were found. The final body length and the SGR increased along with the increase of dietary protein.

Fasted fish showed very low survival rate (2.5%) while in all fed groups the survival rate stayed above 90%. As expected, a clear weight loss and a poorer condition factor index were observed in fasted fish.

Both nitrogen retention and nitrogen intake increased as the level of dietary protein increased (Table 3). The nitrogen retention expressed in percentage of NI recorded the lowest value in fish fed the highest level of protein (60%).

The maximum energy intake was found in fish fed the lowest level of protein (15%) with no significant differences ($p<0.05$) being found among fish fed dietary protein levels above 25% (Table 3). The energy retention in terms of percentage of EI was lower with diets with very low protein content, and no significant differences were found ($p<0.05$) among groups of fish fed diets containing protein levels above 30%.

Concerning fish body composition (Table 4) it was detected that dry matter gradually decreases as the protein levels of diets increase. In contrast, body protein (in percentage of fresh weight) increases linearly with the dietary protein level. The body energy was higher in fish fed protein levels below 35%. Above this level fish exhibit a decrease of body energy, reaching the lowest value with the diet having the highest protein content (Table 4).

Table 2: Growth performance and feed utilization efficiency of fish fed the experimental diets.

	Starvation	15P	20P	25P	30P	35P	40P	45P	50P	55P	60P	SEM
Initial Body Weight (mg)	54.8	54.5	54.5	53.8	53.5	53.5	52.8	53.8	54.5	53.3	53.5	0.34
Final Body Weight (mg)	36.1	219.4 ^{a}	255.2 ^{ab}	278.8 ^{ab}	291.3 ^{ab}	350.5 ^{ab}	350 ^{ab}	389.3^{b}	386.9 ^{b}	383.7 ^{b}	370.9^{b}	14.7
Wet weight gain (mg g ABW^{-1} day^{-1})^{a}	-6.96	20.4^{a}	21.9 ^{ab}	22.9^{abc}	23.3^{abc}	24.9^{bc}	25.0^{bc}	25.7^{c}	25.5^{bc}	25.6^{c}	25.3^{bc}	0.43
Feed intake (mg g ABW-1 day-1)^{b}	-	62.2^{d}	52.5^{cd}	45.0^{bc}	41.3^{ab}	35.3^{ab}	34.5^{a}	34.0^{a}	33.1^{a}	33.5^{a}	34.6^{a}	2.2
Feed efficiency ^{c}	-	0.328^{a}	0.422^{ab}	0.510^{b}	0.566^{bc}	0.704^{cd}	0.724^{cd}	0.754^{d}	0.772^{d}	0.765^{d}	0.733^{cd}	0.04
Protein Efficiency Ratio ^{d}	-	2.11^{cd}	2.22^{d}	2.06^{cd}	1.88^{bcd}	2.01^{cd}	1.77^{abcd}	1.65^{abcd}	1.55^{abc}	1.36^{ab}	1.19^{a}	0.08
Specific Growth Rate ^{e}	-	2.36 ^{a}	2.61^{ab}	2.79 ^{ab}	2.86 ^{ab}	3.18 ^{b}	3.20^{b}	3.35^{b}	3.32^{b}	3.35^{b}	3.27^{b}	0.08
Survival rate (%)	2.5	97.5	92.5	97.5	100	100	100	97.5	92.5	95	95	1.04
Final Body Length (mm)	19.4	26.6^{a}	28.4^{ab}	28.8 ^{bc}	29.2 ^{bcd}	30.7 ^{cde}	30.6 ^{cde}	31.2 ^{e}	31.9^{e}	31.8 ^{e}	31.1 ^{de}	0.42
Condition factor index ^{f}	0.49	1.14	1.07	1.14	1.13	1.21	1.18	1.23	1.18	1.17	1.11	0.02
Length variation coefficient (%) ^{g}	6.1	11.7	9.8	8.3	8.1	6.7	8.5	9.1	7.0	7.7	9.4	0.40

Values presented as mean ± SEM. Different superscripts in the same line were found to be significantly different ($p<0.05$) using the one-way analysis of variance and the Tukey's test ($p<0.05$).

[a] WG=[(individual WG (mg)*1000)/average body weight] / number of days
[b] FI=[(ingested food*1000)/(average body weight/1000)] / number of days
[c] FE=wet weight gain / dry feed intake
[d] PER= wet weight gain / crude protein intake
[e] SGR= [(ln (final individual weight) – ln (initial individual weight)) / number of days]*100
[f] K= (individual final weight*100) / final body length^{3}
[g] LVC= length standard deviation / average length
ABW = average body weight: (initial body weight + final body weight)/2

Table 3: Nitrogen and energy intake and retention of fish fed the experimental diets.

Dietary protein level	Starvation	15P	20P	25P	30P	35P	40P	45P	50P	55P	60P	SEM
N intake (g kg^{-1} ABW day^{-1}) [a]	0.0	1.55^{a}	1.60^{a}	1.79^{ab}	1.99^{bc}	1.98^{bc}	2.26^{cd}	2.49^{de}	2.65^{de}	3.02^{ef}	3.41^{f}	0.14
N retention (g kg ABW^{-1}day^{-1})[b]	-0.32	0.390^{a}	0.440^{ab}	0.492^{abc}	0.532^{bcd}	0.584^{cde}	0.623^{de}	0.647^{e}	0.636^{de}	0.644^{e}	0.654^{e}	0.02
N retention (% N intake) [c]	0	25.3^{abc}	27.7^{bc}	27.6^{bc}	26.8^{bc}	29.5^{c}	27.6^{bc}	26.0^{bc}	24.1^{abc}	21.3^{ab}	19.3^{a}	0.74
E intake (kJ kg ABW^{-1} day^{-1}) [d]	-	1140.7^{c}	959.0^{b}	807.7^{ab}	756.3^{a}	657.7^{a}	628.1^{a}	636.2^{a}	634.5^{a}	632.4^{a}	654.5^{a}	38.6
E retention (kJ kgABW^{-1}day^{-1})[e]	-	205.7	210.6	225.7	219.6	224.1	225.6	226.2	215.7	201.7	198.1	2.79
E retention (% EI) [f]	-	18.1^{a}	22.2^{ab}	28.0^{bc}	29.1^{bc}	34.1^{c}	35.9^{c}	35.6^{c}	34.0^{c}	31.9^{c}	30.4^{bc}	1.33

Values presented as mean ± SEM. Different superscripts in the same line were found to be significantly different ($p<0.05$) using the one-way analysis of variance and the Turkey's test.

[a] NI = [protein intake (g) / (6.25*1000)] / (average body weight* number of days)
[b] NR = [((final body weight * final body protein(%)) – (initial body weight * initial body protein (%)) / 6.25*1000] / average body weight * number of days
[c] NR(%NI) = (NR / NI)*100
[d] EI = (energy ingested * 1000) / (ABW * number of days
[e] ER =[(final weight * final energy FM) – (initial weight * initial energy FM)*1000] / (average body weight * number of days)
[f] ER (%EI) = (ER / EI) * 100
ABW = average body weight: (initial body weight + final body weight)/2

Table 4: Body composition of fish fed the experimental diets.

			Final body composition										
	Initial body composition	**Starvation**	**15 P**	**20 P**	**25 P**	**30 P**	**35 P**	**40 P**	**45 P**	**50 P**	**55 P**	**60 P**	**SEM**
Dry matter (%)	36.28	29.93	35.99 ^{c}	34.53 ^{abc}	35.12 ^{bc}	34.40 ^{abc}	33.63 ^{abc}	33.95 ^{abc}	33.64 ^{abc}	32.63 ^{ab}	31.88 ^{a}	32.20 ^{a}	0.30
Protein (% FM)	23.41	20.56	14.83 ^{a}	14.87 ^{ab}	15.33 ^{abc}	15.96 ^{abcd}	16.02 ^{abcd}	16.78 ^{cd}	16.83 ^{cd}	16.69 ^{bcd}	16.77 ^{cd}	17.22 ^{d}	0.20
Gross energy (kJ g^{-1} FM)	8.46	-	9.67 ^{b}	9.33 ^{b}	9.59 ^{b}	9.29 ^{b}	8.99 ^{ab}	8.97 ^{ab}	8.75 ^{ab}	8.42 ^{ab}	7.73 ^{a}	7.90 ^{a}	0.16

Values presented as mean ± SEM. Different superscripts in the same line were found to be significantly different ($p<0.05$) using the one-way analysis of variance and the Tukey's test.

Based on the assumption that a living organism cannot achieve its maximum theoretical growth potential it was settled that the optimal growth performance of fish (in terms of weight gain, nitrogen retention and energy retention) would correspond to 95% of the theoretical maximum response (Simongiovanni *et al.,* 2012).

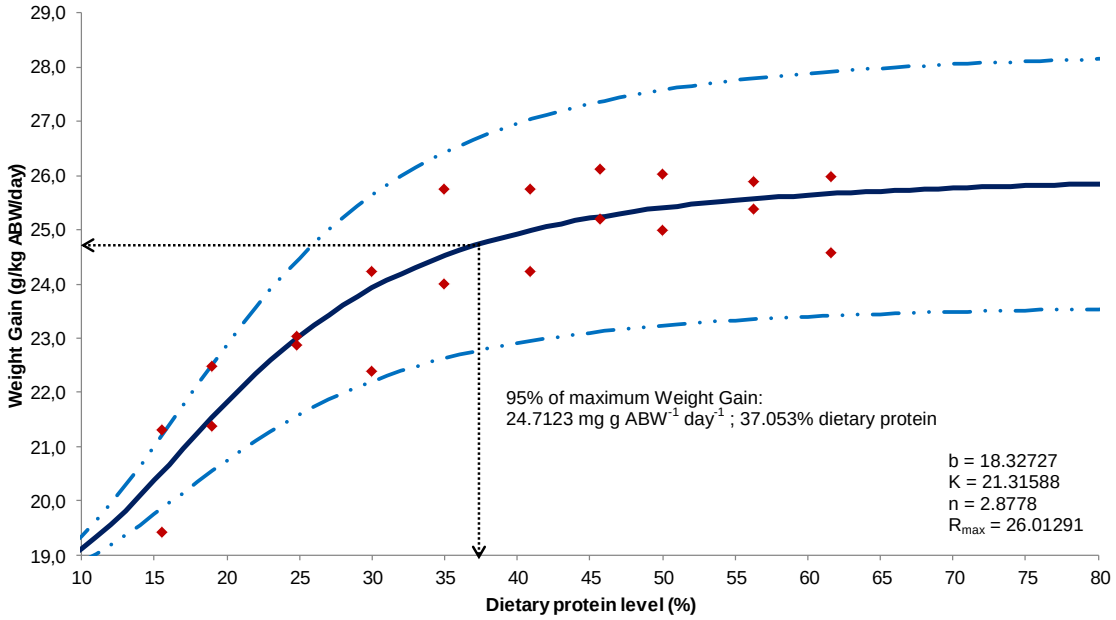

Figure 5: Relationship between the dietary protein (%) and the weight gain (mg g ABW^{-1} day^{-1}) using the 4-SKM model. The R of the model is 0.91826 and it's possible to observe that the maximum WG is 24.7123 mg g ABW^{-1} day^{-1} corresponding to 37.053% of protein in the feed. Upper and lower confidence limits are represented by the dashed lines.

According to the *4-SKM* model was possible to estimate that zebrafish juveniles can reach a maximum weight gain of 24.71 mg g ABW^{-1} day^{-1} (with a confidence interval between the 22.47 mg g ABW^{-1} day^{-1} and 26.95 mg g ABW^{-1} day^{-1}) if they consume a diet containing 37.05% of protein (confidence interval between 32.96% and 39.91%), as can be possible to be seen in Figure 5.

Similarly, observing the Figure 6 it is possible to realize that for a 95% of maximum weight gain the daily nitrogen intake should be 1.99 g kg ABW^{-1} day^{-1} (confidence interval between 1.963 g kg ABW^{-1} day^{-1} up to 2.015 g kg ABW^{-1} day^{-1}). Considering that to reach the maximum estimated growth of 24.71 mg g ABW^{-1} day^{-1} juveniles zebrafish need to ingest 1.99 g of nitrogen kg ABW^{-1} day^{-1}, we can calculate at 33.6 g kg^{-1} the daily amount of a diet containing 37.05% of protein that fish need to ingest for optimal growth.

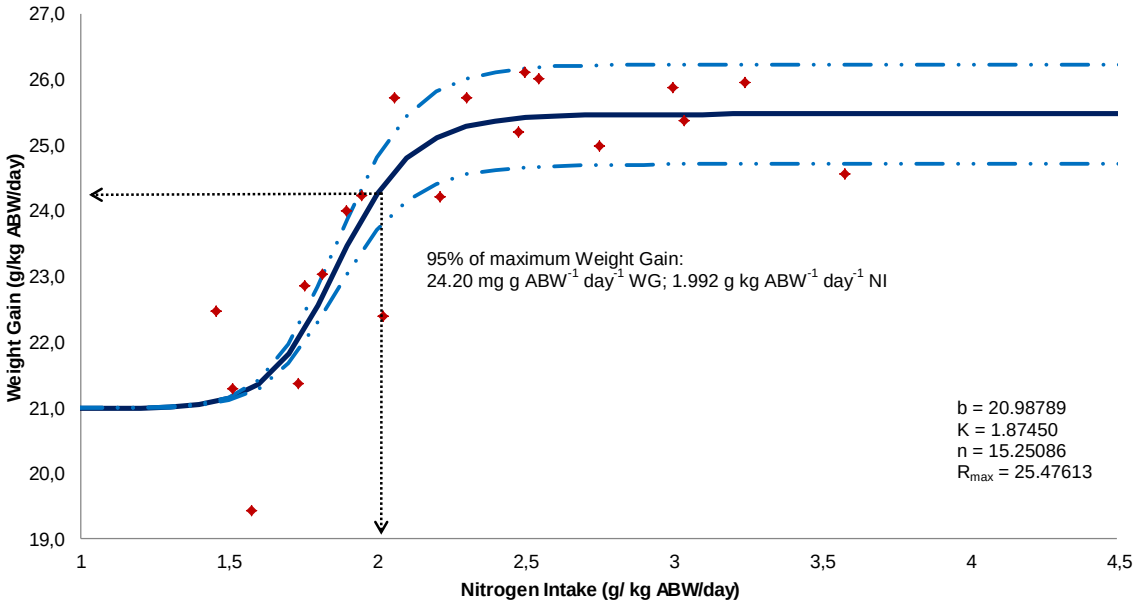

Figure 6: Relationship between the nitrogen intake (g kg ABW^{-1} day^{-1}) and the weight gain (mg g ABW^{-1} day^{-1}) using the 4-SKM model. The R of the model is 0.8804 and it's possible to observe that the maximum WG is 24.2 mg g ABW^{-1} day^{-} corresponding to 1.992 g kg ABW^{-1} day^{-1} of NI. Upper and lower confidence limits are represented by the dashed lines.

Regarding to nitrogen retention, 95% of maximum response was found to be 0.6351 g kg ABW^{-1} day^{-1} (with an interval of confidence between 0.5870 g kg ABW^{-1} $day^{-1}$and 0.6833 g kg ABW^{-1} day^{-1}) what corresponds to a protein level in the diet of 47.05% (with an interval of confidence between 45.63% and 48.21%) as can be observed in Figure 7.

If the purpose is to assure a maximum nitrogen retention in body tissues zebrafish juveniles have to ingest 2.19 g kg ABW^{-1} day^{-1} of nitrogen (values of confidence between 2.09 g kg ABW^{-1} day^{-1} and 2.41 g kg ABW^{-1} day^{-1}) to retain 95% of the theoretical maximum nitrogen retention (0.6152 g kg ABW^{-1} day^{-1}), as can be seen in Figure 8. Reasoning as above, we can calculate at 29.1 g kg^{-1} the daily amount of a diet containing 47.05% of protein that fish need to ingest for optimal nitrogen retention.

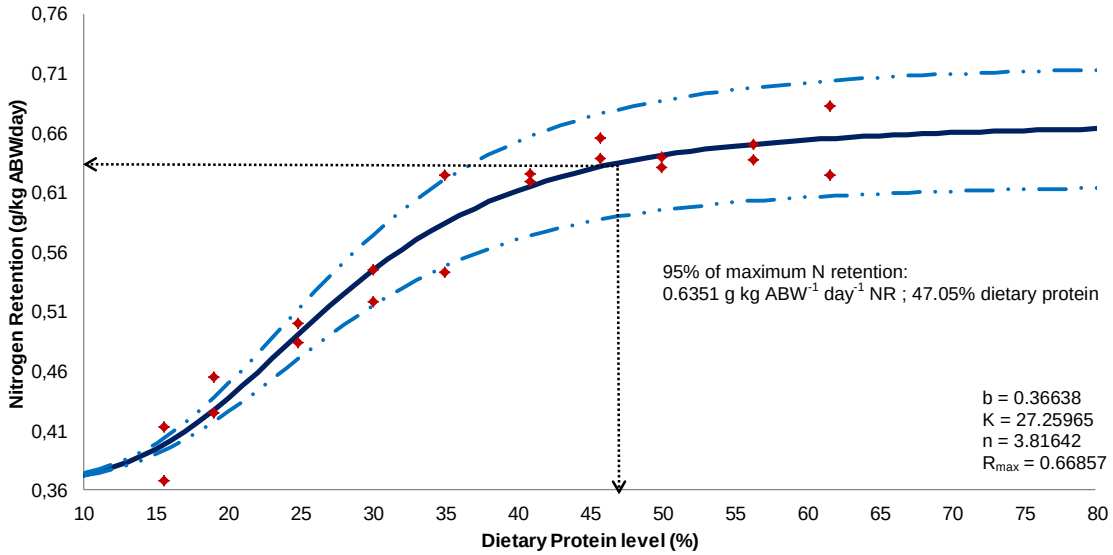

Figure 7: Relationship between the dietary protein (%) and the nitrogen retention (g kg ABW^{-1} day^{-1}) using the 4-SKM model. The R of the model is 0.97399 and it's possible to observe that the maximum NR is 0.6351 g kg ABW^{-1} day^{-1} corresponding to 47.05% of protein in the feed. Upper and lower confidence limits are represented by the dashed lines.

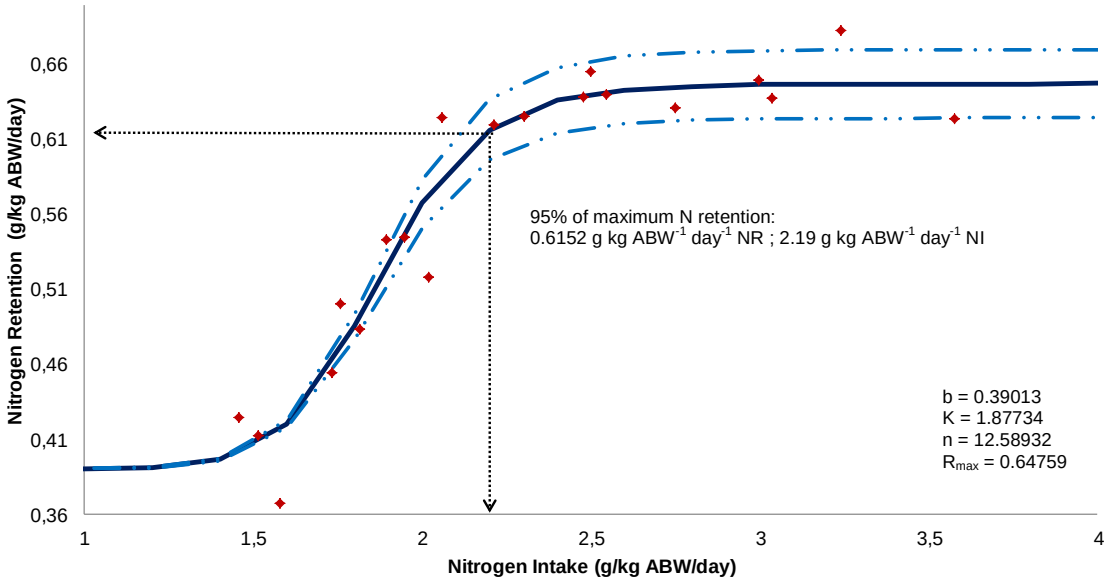

Figure 8: Relationship between the nitrogen intake (g kg ABW^{-1} day^{-1}) and the nitrogen retention (g kg ABW^{-1} day^{-1}) using the 4-SKM model. The R of the model is 0.96626 and it's possible to observe that the maximum NR is 0.6152 g kg ABW^{-1} day^{-} corresponding to 2.19 g kg ABW^{-1} day^{-1} of NI. Upper and lower confidence limits are represented by the dashed lines.

In which concerns energy retention, by plotting energy retention as percentage of energy intake against the protein to energy ratio (P/E) of different diets (Figure 9) we can clearly see a maximum energy retention of 38.088 kJ kg ABW^{-1} day^{-1} (values within

a confidence interval between 28.537 kJ kg ABW $^{-1}$ day^{-1} and 41.637 kJ kg ABW $^{-1}$ day^{-1}) corresponding to a P/E ratio of 24 g MJ^{-1} (with both lower and upper confidence values corresponding also to 24 g MJ^{-1}) and lower values of retention for both lower and higher P/E ratios.

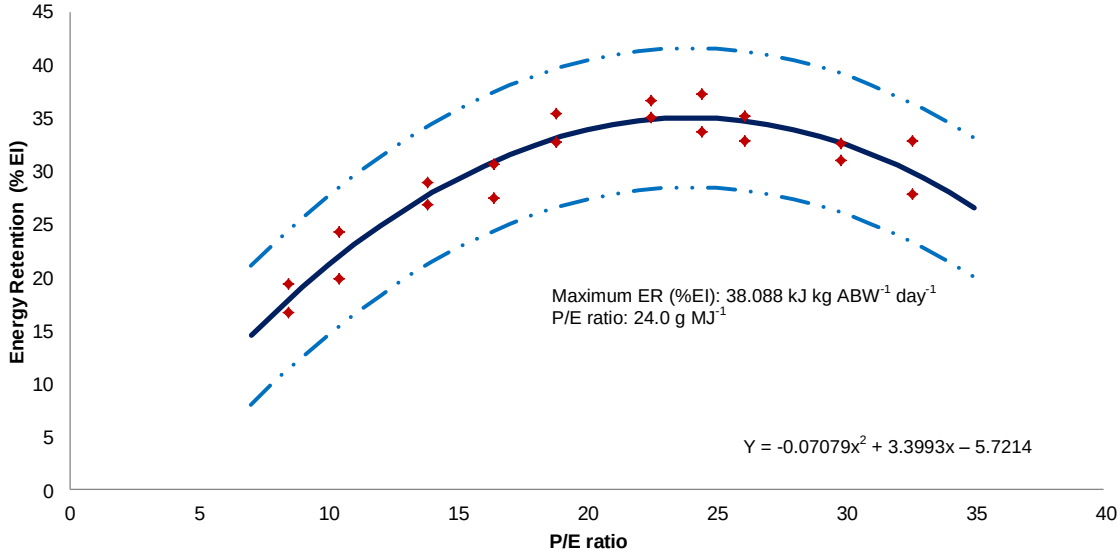

Figure 9: Second-order polynomial model applied to ER (%EI) in order to P/E ratio of the diets. The R obtained was equal to 0.95208 and the maximum plateau of ER reached was 38.088 kJ kg ABW^{-1} day^{-1} corresponding to a P/E ratio of 24 g MJ^{-1}. Upper and lower confidence limits are also represented by the dashed lines.

Relating the energy retention to the percentage of crude protein in the diet (Figure 10) we can observe that 45% of dietary protein will lead to a maximum energy retention of 35.286 kJ kg ABW $^{-1}$ day^{-1} (with confidence interval settled between 29.013 kJ kg ABW $^{-1}$ day^{-1} and 41.558 kJ kg ABW $^{-1}$ day^{-1}). Therefore lower levels of dietary protein resulted in low energy retention even though that fish have ingested a greater amount of food (Table 2) and, consequently, higher amount of energy. For juveniles fed with protein levels above 30% a linear improvement of the energy retention along with the protein increment wasn't observed what point towards the fact that they reached a maximum of ER (Figure 11). Gathering the information presented in Figures 7 and 10 it becomes evident that a dietary protein level between 45% and 47% optimize simultaneously nitrogen and energy retention.

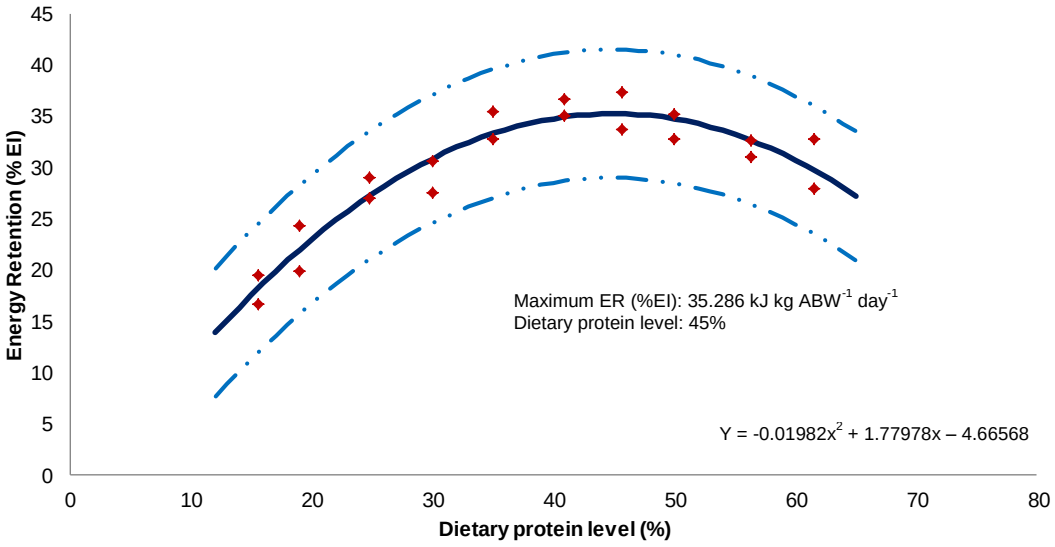

Figure 10: Second-order polynomial model applied to the energy retention data (% EI) in relation to the dietary protein level (%). The correspondent R is 0.95247 and the ER maximum plateau is 35.286 kJ kg^{-1} day^{-1} being equivalent to 45% of dietary crude protein. Upper and lower confidence limits are also represented by the dashed lines.

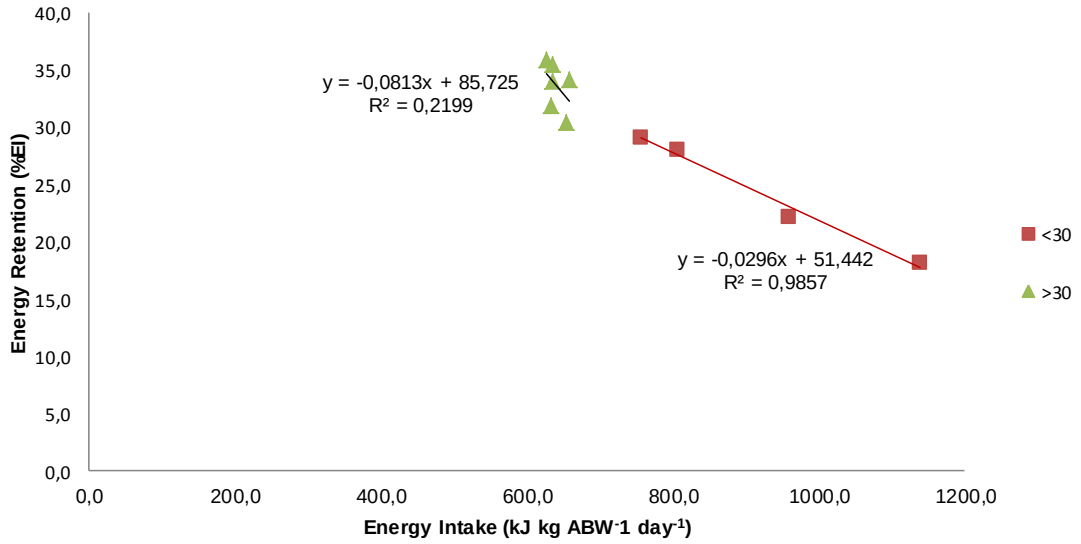

Figure 11: Relationship between Energy Intake and Energy Retention.

1.4. Discussion

Growth performance, body composition and nitrogen and energy retention of zebrafish juveniles were all greatly influenced by dietary protein levels. As for the

generality of fish species, this makes clear that it is of primary importance to take into account the protein content in diets for zebrafish juveniles, in order to promote optimal growth and adequate physiological condition. Besides the protein level, protein source also plays a key role in growth and body composition of fish, including zebrafish (Smith *et al.,* 2013), mainly due to the amino acid profile.

The estimated optimal dietary protein level that can lead to the maximum growth in the juvenile phase of *D. rerio* was settled at 37.05% corresponding to a weight gain of 24.7 mg g ABW^{-1} day^{-1}. Moreover, it was also determined that a daily nitrogen intake of 1.99 g kg ABW^{-1} day^{-1} will result in the maximum weight gain. Combining these data was possible to estimate that the daily amount of feed necessary to obtain the best growth is 33.6 g kg^{-1} with a diet constituted by 37.05% of protein. This is important information that can provide an easier and more practical approach to feeding practices on the rearing of zebrafish juveniles. Similarly, in terms of nitrogen retention was obtained a maximum of 0.6 g kg ABW^{-1} day^{-1} matching a crude protein level of 47.05% and a daily nitrogen intake of 2.19 g N kg ABW^{-1} day^{-1}. The combination of these values indicate that using a diet containing 47% of protein the juveniles have to be fed with a ration of 29.1 g kg^{-1} on a daily basis to obtain maximum nitrogen retention.

A maximum energy retention of 35.286 kJ kg ABW^{-1} day^{-1} (expressed as percentage of energy intake) was obtained with a dietary protein level of 45% of crude protein and a P/E ratio of 24 g MJ^{-1}. Thus, our results support that a dietary protein level ranging between 45 – 47% will allow simultaneously maximum energy and nitrogen retention.

As already mentioned, it has been suggested that until specific studies on the nutritional needs of zebrafish are available, zebrafish requirements could be derived from those available for other cyprinids species (Lawrence , 2007; Watts *et al.* , 2012). In fact, protein requirements of different cyprinids species have been evaluated. Thus, in the Indian carp *Labeo fimbriatus* a digestible protein requirement about 30% was determined for fingerling growth (Jena *et al.*, 2012). Similarly, Islam & Tanaka (2004) tested different protein levels for mahseer (*Tor putitora*) fingerlings and concluded that the optimum dietary protein level for maximal growth ranged between 45 and 50%. Also, a study in Jian carp juveniles Liu *et al.* (2009) estimated the need of a dietary protein level of about 34% to achieve the best growth performance. For the mrigal carp *Cirrhinus mrigala* the protein requirements before reaching the adult stage vary

between 30 and 45% and for the common carp *Cyprinus carpio* the protein needs range between 34 – 37% in the juvenile stage and 28 – 32% in the adulthood (FAO, 2014). Taking into account all these values, protein requirements that we estimated for zebrafish juveniles in the present study – 37% for maximal weight gain and 47% for maximal nitrogen retention – fall within the general range found for other cyprinid species.

A widespread practice in the husbandry of zebrafish is utilising commercial dry feeds that can be simply purchased in any ornamental fish store. In the light of our results, the most commonly used of these feeds (*TetraMin*) with around 47% of protein (information from the package) meet the protein requirements of zebrafish juveniles.

Regarding food consumption, we found that zebrafish juveniles fed diets with lower protein levels ingested more food, and consequently more energy, than the ones fed diets with higher protein levels. This finding is in accordance with Laeger & Morrison (2013) about the central influence of dietary protein on food consumption and metabolism. It also agrees with Coutinho *et al.* (2012) that found an increase of food intake of sharpsnout sea bream (*Diplodus puntazzo*) as the dietary protein level decreased. The hyperphagic response observed in this experiment for juveniles exposed to protein levels below 30% may have occurred because these organisms ingested more food with the purpose to fulfil their protein requirements. However, this issue is not completely clear. According to Morrison *et al.* (2012), variations in the food consumption related with the dietary protein level may also result from the fact that diets with lower protein content often contain higher carbohydrates content, and the increased intake in low-protein diet can be related to this increase in carbohydrate content rather than with the reduction of protein. Our results also suggest that zebrafish juveniles fed diets with protein levels below 30% were not able to regulate energy intake, consuming more energy than they could retain. Contrarily, fish fed diets with protein levels above 30% seemed to be able to regulate energy intake.

Regarding body composition, there was a clear linear protein increment in the carcass as the dietary protein level increased. Such evidence meets the findings of Islam & Tanaka (2004) that observed an increase of body protein of the cyprinid *Tor putitora* as the dietary protein also increased. Similar results were also found by Jena *et al.* (2012). On the other hand, body water content increased along with the dietary protein content, as it was also observed also for Jian carp (Liu *et al.*, 2009). Likewise

Hernández *et al.* (2001) also refer that body dry matter content was higher in shasnout seabream fed diets containing lower protein/energy ratio.

2. Evaluation of the effect of dietary protein levels on post-juvenile growth and reproductive performance of zebrafish

2.1. Introduction

The adult stage of zebrafish is reached by the third month of development, when individuals measure more than 18 mm of SL and are viable to reproduce (Singleman & Holtzman, 2014). During this stage the somatic growth slows while the gonadal growth accelerates (Hardy & Barrows, 2002). Adult sexually mature zebrafish under good nutritional conditions are of the most importance for laboratories that need to obtain viable fertilized embryos (Gonzalez Jr., 2012). Furthermore, even if zebrafish is most utilized in the larval stage, the utilization of individuals in juvenile and adult stages has similarly been growing over time (Singleman & Holtzman, 2014).

The importance of broodstock nutrition in the good quality of fish eggs has been well recognized, and especially female nutrition is considered fundamental in egg composition and later survival of offspring (Lupatsch *et al.*, 2010). A good-quality egg can be defined as its capability to be fertilized and subsequently develop into a healthy individual, and the reproductive success of a fish species can be measured by the capacity to produce high amount of good-quality eggs. Characteristics such as egg morphology, egg biochemical composition and egg hatchability (Rodríguez-González *et al.*, 2006) can be used to evaluate egg quality. Given that the reproductive performance is a good indicator of the quality of a diet to rear sexual mature individuals (Kaushik *et al.*, 2011) this second experiment was primarily carried out to evaluate the effect of dietary protein level on the reproductive performance of zebrafish early adults. Furthermore, it was evaluated the effect of dietary protein level on zebrafish growth and body composition in the post-juvenile phase, characterized by a much lower growth rate than the juvenile phase, and whether males and females are differently affected.

2.2. Materials and methods

2.2.1. Experimental procedure

Post-juveniles zebrafish aged between 152 and 207 *dpf* at the beginning of the experiment were obtained as referred in 1.1.2. Following the methodology for zebrafish sex determination proposed by Yossa *et al.* (2013), males and females were identified by visual recognition of the absence (males) or presence (females) of genital papilla. This structure typically measure between 0.1 to 0.4 cm and is visually transparent, covering the urogenital opening of mature females, allowing the sex recognition without damaging the animals (Yossa *et al.*, 2013). A sample of 20 fish from the stock were euthanized with phenoxiethanol (0.5 mL/L) and frozen and stored for later analysis of body composition.

The experimental system was the same used in the previous experiment (see 1.1.1), but this time experimental units consist of 4-L plastic tanks. Experimental conditions were also maintained as in the first experiment (see 1.1.1). A total of 16 experimental groups were established. Each group was formed by three females and three males placed together inside an experimental unit, after being weighed separately. Four isoenergetic diets with protein levels of 30, 40, 50 and 60% were tested, and each diet was randomly assigned to quadruplicate experimental groups. Diets are the same used in the first experiment and their nutritional composition is presented in Table 1. In this experiment we chose to use four replicates for each dietary treatment due to the high variability in the reproductive performance of zebrafish. On the other hand, consequently, we had to use only four dietary treatments due to space constraints.

Fish were fed to apparent satiation twice daily, six days a week, throughout a period of 47 days. At the end of this period, each experimental unit was prepared for fish reproduction during the next 18 consecutive days. For that, each day in the late afternoon, a plastic net with some glass marbles was placed above the bottom of the tanks (Figure 12) to induce spawning. The next morning, one hour after light turned on, tanks were inspected for the presence of eggs and eggs released by females were collected. Eggs collected during the first twelve days were preserved in ethanol 70% for posterior counting. Eggs collected the following six days were stored for later analysis of protein content.

Twenty eggs from a single spawn in each experimental unit were incubated in multi-well plates with water from an independent reservoir, inside an incubation chamber at the same temperature and photoperiod as in the recirculation system. After three days post-fertilization the number of newly-hatched larvae was counted for evaluation of the hatching rate. After seven days post-fertilization surviving larvae were euthanized and photographed with a digital camera coupled to a stereomicroscope. Digital images (Figure 13) were used to measure fish standard length (from snout until the posterior tip of the notochord) with the software *ImageJ* (available at *http://imagej.nih.gov/ij/index.html*).

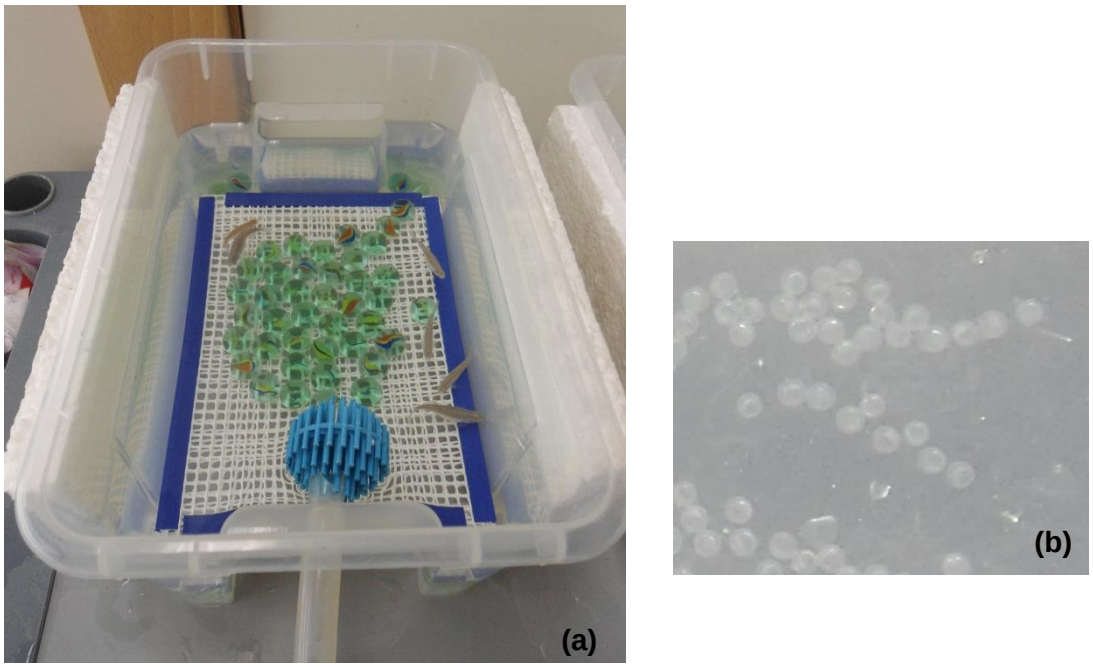

Figure 12: Tank prepared for spawning induction of zebrafish post-juveniles (a). Fertilized eggs at tank bottom (b). Photographs taken by Helena Fernandes.

At the end of the growth and reproduction periods all fish were euthanized with phenoxiethanol (0.5 mL/L), weighed and measured as described for the first experiment (see 1.1.3), and frozen for later analysis of body composition.

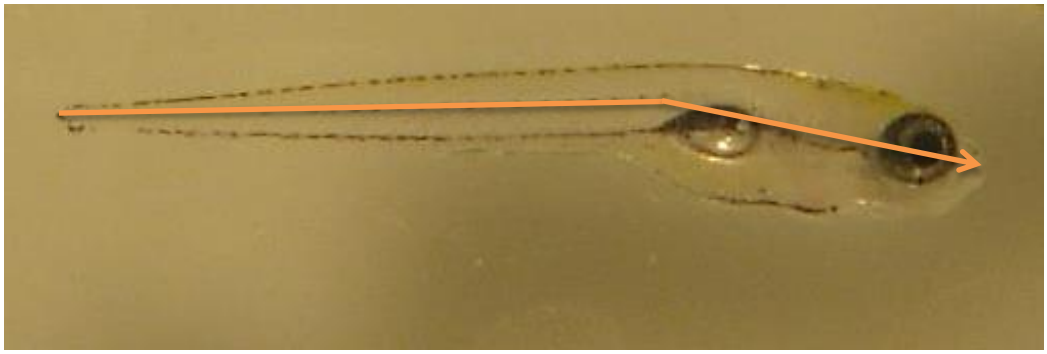

Figure 13: Larva 7 *dpf* for measurement of standard length. Photograph taken by Helena Fernandes.

2.2.2. Chemical analysis

Chemical analyses of fish body composition were performed by the same methods referred for the previous experiment (see 1.1.4).

Protein content of zebrafish eggs was analysed by the Bradford method. Eggs were firstly lyophilized during approximately 19 hours and then homogenised with NaOH 0.5 N. The homogenate was centrifuged at 3000 *rpm* for 10 minutes at 20˚ C, and the optical density of the supernatant read at 595 nm in a microplate reader.

2.2.3. Statistical analysis

From data directly collected during the experiment (initial and final fish weight and fish length, food consumed, chemical composition of diets, and initial and final fish body chemical composition) were derived the following parameters indicative of fish growth performance, feed efficiency utilization, and nitrogen intake and retention: Weight Gain, Specific Growth Rate, Feed Efficiency, Protein Efficiency Ratio, Nitrogen Intake, Nitrogen Retention. The following parameters indicative of the reproductive performance were also evaluated from collected data: average number of eggs per spawning event, number of eggs per female body weight, hatching rate, egg protein content and larval length.

Whenever it was possible to obtain separate data for males and females, such as for growth performance and body composition, data were analysed by two-way analysis of variance (ANOVA), in order to investigate not only the effect of dietary

protein level but also the effect of gender. Otherwise, data were analysed by one-way ANOVA. In all cases, analyses were performed using the *IBM SPSS Statistics 22 software*. If significant differences were found ($p<0.05$) the Tukey multiple range test was used to discriminate means. All data were checked for normal distribution and homogeneity of variance and when needed they were transformed.

2.3. Results

In this experiment it was detected that the frequency of spawning was very irregular in all replicates and irrespective of the dietary treatment.

Even without statistical differences among the reproductive indicators ($p<0.05$), except for the larval length, both the number of eggs per female body weight and the average number of fertilized eggs, was greater in fish fed with 40% of protein (Table 5). Only the hatching percentage showed to be higher in the 60% dietary protein treatment. For larval length was found a statistical difference among dietary treatments, with the smallest larvae resulting from progenitors fed the diet with highest protein content (60%) and the largest larvae resulting from progenitors fed the diet with lowest protein content (30%) (Table 5).

Table 5: Indicative parameters of the reproductive performance of zebrafish adults fed the experimental diets.

Dietary protein level (%)	30 P	40 P	50 P	60 P	SEM
Average number of eggs per spawning event	42.3	142.1	87.0	114.4	17.6
Number of eggs number per female body weight (eggs BW^{-1})	250.7	608.0	382.5	521.2	82.4
Hatching rate (%)	38.8	49.8	46.3	57.5	6.86
Egg protein content (% DM)	63.17	63.27	58.48	63.69	0.87
Larval length (mm)	4.12 ^{c}	3.92 ^{ab}	4.03 ^{bc}	3.89 ^{a}	0.016

Values presented as mean ± SEM. Different superscripts in the same line were found to be significantly different ($p<0.05$) using the one-way analysis of variance and the Turkey's test.

It was observed that both weight and length of adults were strongly gender dependent, with females being larger and having about twice the weight of males (Table 6). However, no significant differences were found between males and females in terms of growth performance expressed both as weight gain and growth rate (Table 6).

Observing Table 7 it is possible to notice that feed intake tends to decrease as the dietary protein level increased, but no significant differences were found ($p<0.05$). Contrarily, feed efficiency tends to increase as the protein percentage of the diets increased, but again with no significant differences found ($p<0.05$). Significant differences ($p<0.05$) were found for nitrogen intake, with fish fed higher levels of dietary protein ingesting more nitrogen than fish fed lower levels of dietary protein. No trends were observed regarding PER or nitrogen retention.

Table 6: Growth performance of zebrafish adults fed the experimental diets

Sex	**Females**				**Males**				**SEM**
Dietary Protein Level	**30 P**	**40 P**	**50 P**	**60 P**	**30 P**	**40 P**	**50 P**	**60 P**	
Initial body weight (mg)	578.3	586.7	588.3	580.8	285.8	292.5	285	309.2	27.9
Final body weight (mg)	695.4	776.7	773.1	783.3	345.8	379.2	372.9	425.8	36.4
Weight gain (mg g ABW^{-1} day^{-1})	7.09	10.32	10.24	10.99	7.02	9.68	9.44	11.64	0.56
Specific Growth Rate	0.72	1.06	1.05	1.13	0.71	0.99	0.97	1.20	0.06
Final body length (cm)	3.8	4.1	4.0	4.0	3.4	3.6	3.5	3.5	0.05

	Variation source		
	Protein	**Sex**	**Interaction**
Initial body weight (mg)	-	***	-
Final body weight (mg)	ns	***	ns
Weight gain (mg g ABW^{-1} day^{-1})	ns	ns	ns
Specific Growth Rate	ns	ns	ns
Final body length (cm)	ns	***	ns

Values presented as mean ± SEM.

*** - significant, $p<0.001$; ns - non-significant; $p>0.05$.

Table 7: Feed utilization, nitrogen utilization and nitrogen retention of zebrafish adults fed the experimental diets.

Dietary protein level (%)	30P	40 P	50 P	60 P	SEM
Feed Intake (mg g ABW^{-1} day^{-1})	38.23	35.48	31.24	31.96	1.13
Feed Efficiency	0.19	0.29	0.30	0.34	0.02
Protein Efficiency Ratio	0.63	0.70	0.60	0.55	0.04
N Intake (g kg ABW^{-1} day^{-1})	1.84 ^{a}	2.32 ^{ab}	2.50 ^{ab}	3.15 ^{b}	0.13
N Retention (g kg ABW^{-1} day^{-1})	0.13	0.21	0.18	0.23	0.02
N Retention (% NI)	7.20	9.22	7.04	7.35	0.73

Values presented as mean ± SEM. Different superscripts in the same line were found to be significantly different ($p<0.05$) using the one-way analysis of variance and the Turkey's test.

As can be observed in Table 8, dry matter for males and females decreased as the protein content of feed increased, even if no statistical differences were found neither in order to dietary protein or gender. Body protein (in percentage of fresh weight) was not affected by the dietary protein treatment in both genders.

Table 8: Body composition of zebrafish adults fed the experimental diets.

Sex	**Females**				**Males**				**SEM**
Dietary Protein Level	**30 P**	**40 P**	**50 P**	**60 P**	**30 P**	**40 P**	**50 P**	**60 P**	
Dry matter (%)	27.49	26.25	24.86	24.48	25.79	25.34	22.94	22.64	0.41
Body protein (% FW)	14.95	15.06	15.19	16.09	14.84	15.16	14.20	14.18	0.19

	Variation source		
	Protein	**Sex**	**Interaction**
Dry matter (%)	ns	ns	ns
Body protein (% FW)	ns	ns	ns

Values presented as mean ± SEM.

ns - non-significant; p>0.05.

2.4. Discussion

Females fed with diets with the lowest protein levels were able to spawn and produce eggs in the same way as females fed higher dietary protein levels. Moreover, eggs showed high protein content irrespectively of the protein level of diet ingested by the broodstock, and no correlation was found between larval size and dietary treatment. All these results indicate that zebrafish continue to spawn and produce good-quality eggs that originate viable healthy larvae even under restricted feeding conditions. A similar strategy was suggested for tilapia (*Oreochromis niloticus*) by Lupatsch *et al.* (2010). However these authors alert that maintaining this situation for a long period of time will compromise total fecundity and spawning intervals. This ability to keep production of good-quality eggs under sub-optimal feeding conditions is done by sacrificing the somatic growth, channelling nutrients to produce eggs with a similar quality as females under optimal nutritional conditions (Lupatsch *et al.*, 2010).

Female zebrafish achieved a weight two-fold higher than males. This agrees with findings of Gonzalez Jr. (2012) that zebrafish females grew more and faster than males.

Although there was a trend for a higher feed intake when fish were fed diets with lower protein content, no significant differences in weight gain were found comparing fish fed with higher and lower protein levels. This means that fish at this post-juvenile phase were able to compensate the lower quantity of protein in the diet by increasing feed intake, keeping growth at a normal rate, contrarily to what was observed at the juvenile phase. Differences between both stages can be explained by lower protein requirements at the post-juvenile stage, which may be related with a growth slowdown at this phase. Fournier *et al.* (2002) also reported an inverse relationship between the nitrogen level of the diets and the feed intake, concluding that fish increased food consumption in order to compensate the lower levels of protein in diets.

Overall results suggest that dietary protein levels as low as 30% can be used at the post-juvenile stage of zebrafish without negative impacts on reproductive performance and growth.

Conclusions

Considering the aims of the present study, our main conclusions are:

- Dietary protein requirements for zebrafish juveniles were estimated at about 37% for maximum weight gain, 47% for maximum nitrogen intake, and 45% for maximum energy retention.
- A daily ration of 33.6 g kg^{-1} of a diet containing 37% of protein and a daily ration of 29.1 g kg^{-1} of a diet containing 47% of protein will promote, respectively, maximum weight gain and maximum nitrogen retention in zebrafish juveniles.
- Neither growth nor the reproductive performance of zebrafish post-juveniles was significantly influenced by dietary protein levels between 30% and 60%, suggesting that relatively low dietary protein levels can be used in zebrafish husbandry, at this stage.

In our opinion, overall results should be viewed as a contribution towards the formulation of standard diets for zebrafish juvenile and post-juvenile stages, aiming the general standardization of zebrafish husbandry.

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