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Faculdade de Ciências e Tecnologia

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Dissertação apresentada para a obtenção do grau de Mestre em Aquacultura e Pescas com especialização em Aquacultura

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Sob a supervisão de:

Doutor Paulo J. Gavaia
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“The ability to replicate research is fundamental for good science”

(Dennis E. Barnard)

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Durante todo o meu percurso académico passei por determinadas experiências que me permitiram ser a pessoa que sou hoje. Um obrigado a todos os que deram o seu contributo.

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RESUMO/ABSTRACT

Resumo

O peixe zebra (*Danio rerio*) é considerado uma espécie modelo com um baixo custo de produção e adequada para investigação científica, devido à sua fácil manutenção e ao seu sucesso reprodutivo. Actualmente, devido à vasta informação acerca do seu genoma, função de genes estabelecida, e ferramentas para manipulação da expressão de genes, o uso desta espécie como modelo tem vindo a aumentar. No entanto, os resultados científicos podem ser comprometidos devido aos escassos estudos feitos acerca dos requisitos nutricionais desta espécie. O desenvolvimento de dietas equilibradas é uma necessidade, de forma a possibilitar a replicação de resultados experimentais e permitir um melhor bem-estar animal. Este estudo teve como objectivo a avaliação dos efeitos no peixe zebra da modelação nutricional em vitaminas (vitamina D3), fosfolípidos (fosfatidiletanolamina e fosfatidilcolina) e minerais (selénio, zinco, manganês e iodo) suplementados em dietas purificadas. Esta avaliação foi efectuada em relação ao crescimento e sucesso reprodutivo dos adultos e ao crescimento, sobrevivência e avaliação do desenvolvimento esquelético das larvas. Para tal, foram utilizados grupos de 8 reprodutores por tratamento (5 fêmeas e 3 machos), que foram alimentados durante 28 dias com cada uma das dietas experimentais, sendo depois reproduzidos. Como resultados, foi observado que a dieta comercial controlo (CD) apresentou efeitos positivos no crescimento dos adultos, mas sem qualquer sucesso reprodutivo. O grupo com suplemento em calciferol (vitamina D3) apresentou os valores mais baixos em termos de crescimento e peso dos adultos e baixos valores de TM do esperma assim como uma baixa taxa de eclosão dos ovos. O incremento de fosfatidilcolina (PC) levou a um aumento do crescimento dos adultos e das larvas assim como a um aumento do diâmetro do ovo e do espaço perivitelino. O incremento em fosfatidiletanolamina (PE) na dieta teve um efeito positivo nos parâmetros de qualidade dos reprodutores com um aumento na mobilidade total (TM) e progressiva (PM) do esperma, diâmetro do ovo e incremento do espaço perivitelino, mas com um baixo crescimento das larvas. O suplemento em zinco (ZN) teve como efeitos um aumento inicial no crescimento larvar (5dpf) e da deposição mineral total. Com o incremento de iodo (I) na dieta formam observados bons resultados de crescimento larvar (28 dpf) mas com baixos valores em termos de diâmetro do ovo, espaço perivitelino e deposição mineral.

Palavras-chave: Peixe-zebra, requisitos nutricionais, fosfatidilcolina, fosfatidiletanolamina, zinco, iodo, vitamina D3, mobilidade esperma, desenvolvimento esquelético.

Abstract

Zebrafish (*Danio rerio*) is considered a cost-effective model species adequate for research, due to easy maintenance and breeding. Nowadays the use of this species is increasing mainly due to the information coming from genome sequencing, known target genes, and technology for gene knock–down and manipulation. However, the nutrition of zebrafish is poorly studied, with the fish being fed with variable sources of artemia nauplii and commercial diets, which cause effects in research results. The necessity for an adequate balanced diet is a need for scientific replication and animal welfare. The aim of this study was to evaluate the effect of nutritional modulation by supplementing purified diets in vitamins (vitamin D3), phospholipids (phosphatidylethanolamine - PE and phosphatidylcoline - PC) and trace minerals (selenium, zinc, manganese and iodine) in zebrafish growth and reproductive success and subsequently in the larval growth, survival and skeletal development. The experiment was performed with groups of 8 adult zebrafish (5 females and 3 males) per dietary treatment, which were fed with each experimental diet during 28 days before being mated. As results it was observed that control diet (CD) had a positive effect on breeder's growth and weight, but without success in reproduction. The vitamin D3 - cholecalciferol (D3) presented the lowest growth in adult's weight, and the lowest TM. The PC diet caused an increment in adult length and an increase in egg diameter and perivitelline space, but with a negative effect in larvae length. The increment in PE on diet of adult zebrafish improved sperm total (TM) and progressive motility (PM), egg diameter and led to an increase of perivitelline space, but with poor results in larvae length. The zinc (ZN) diet caused an increment in initial larval growth (5dpf) and total mineral deposition. The increment of iodine (I) on diet led to a good larvae growth in length (28 dpf), but presented lower values in of egg diameter, perivitelline space. This treatment, despite the poor indicators of egg quality, induced a higher larval growth and a higher mineral content.

Keywords: Zebrafish, nutritional requirements, phosphatidylcholine, phosphatidylethanolamine, zinc, iodine, vitamin D3, sperm motility, skeletal development.

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List of Abbreviations

Kg - Kilogram
g - Gram
L - Litre
dl - decilitre
ml – Millilitre
µl – Microlitre
m - Meter
cm - centimetre
mm – Millimetre
µm - Micrometre
h - Hour
min - minutes
s- Second
µS – electrical conductivity measurement
UI – international units
Ppt – parts per thousand
DM – dry matter
DW – dry weight
°C – temperature measurement in Celsius degrees
hpf – hours post fertilization
dpf – days post fertilization
TL – total length
SL – standard length
IBW – weight gain
DGI – daily growth index
ABW – average body weight
CF – condition factor
SGR – specific growth rate
PBS – phosphate buffer saline
CD – Commercial diet / control diet
PURE – purified diet
PE – supplemented purified diet with Phosphatidylethanolamine
PC - supplemented purified diet with Phosphatidylcholine
PI – phosphatidylinositol
PS – phosphatidylserine
SE - supplemented purified diet with Selenium
MN - supplemented purified diet with oxide manganese
ZN - supplemented purified diet with oxide zinc
I - supplemented purified diet with iodine
D3 - supplemented purified diet with Cholecalciferol / vitamin D3
Ara – arachidonic acid

PUFAs – polyunsaturated fatty acids
TM – total motility
PM – Progressive motility
VCL – Curvilinear velocity
VSL – Straight line velocity
LIN – Linearity
CASA – computer assisted sperm analysis software
ROS – reactive oxygen species
Def – deformed fish
Parhyp. – parhypural(s)
Hyp. – Hypural(s)
AV C – Abdominal vertebra (centra)
AV N - Abdominal vertebra (neural arch)
AV H - Abdominal vertebra (hemal arch)
CV C – Caudal vertebra (centra)
CV N - Caudal vertebra (neural arch)
CV H - Caudal vertebra (hemal arch)
CFV C – Caudal fin vertebra (centra)
CFV N – Caudal fin vertebra (neural arch)
CFV H – Caudal fin vertebra (hemal arch)
CF Parhyp. /Hyp 1-2 – Caudal fin parhypural and hypurals 1 and 2
CF Hyp. 3-5 – Caudal fin hypural 3, 4 and 5
THs – tyroid hormones
BOP – basioccipital articulatory process
TALENs - transcription activator-like effector nucleases
ZFNs – zinc – finger nucleases

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INTRODUCTION

1. Introduction

1.1 The zebrafish model

The relevance of model species on research has been growing in the past century due to its high applicability not only on human health but also for different industries such as in the pharmaceutical and aquaculture sectors (Robles et al., 2009; Ulloa et al., 2011).

Model species must be practical for manipulation, with physiological tractability and fundamentally, must have high extrapolation for human applications (Ribas and Piferrer, 2013). Some of the most common animal models are the nematode worm (*Caenorhabditis elegans*), fruit fly (*Drosophila melanogaster*), Medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), amphibian African clawed toad (*Xenopus laevis*) and the mouse *(Mus musculus*) (Rubio-Aliaga, 2012). Recently, the use of zebrafish has been growing on different areas of research (Table 1) (Ribas and Piferrer, 2013) due to its inherent advantages like the short life cycle, high number of eggs, big embryos and external fertilization with rapid development and high resistance to experimental research (Westerfield, 2000; Lawrence, 2007; Ulloa et al., 2011). Additionally, zebrafish are easy to maintain and breed in captivity, facilitating is production in laboratory. Due to the high homology between some relevant zebrafish and human genes, it has been successfully used to study human genetic diseases (Howe et al., 2013). Zebrafish small size and the phylogenetic distance towards humans consist in this species major disadvantages (Ribas and Piferrer, 2013). Until now there were a lack of knockout technology, which recently started to be developed through a high throughput targeted mutagenesis protocols with inactivated genes using zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (Sood et al., 2013).

Zebrafish (*Danio rerio*) is a tropical freshwater teleost from the Cyprinidae family and Rasborinae subfamily (Meyer et al., 1993; Nasiadka and Clark, 2012; Ribas and Piferrer, 2013). This species is distributed by several south Asian countries (India, Bangladesh, Nepal, Myanmar and Pakistan) (Spence et al., 2008) with a monsoon climate.

Table 1 - Number of publications for some of the most used model fish species worldwide (adapted from Ribas and Piferrer, 2013).

	Zebrafish (*Danio rerio*)	Goldfish (*Carassius auratus*)	Medaka (*Oryzias latipes*)	Pufferfish (*Takifugu rubripes*)
Development	10002	878	1157	130
Growth	3469	1307	461	69
Stem cells	1494	177	130	13
Stress	1037	331	173	24
Cancer	1021	33	87	11
Genetics	703	21	124	26
Immune	654	146	81	58
Genomics	446	16	108	74
Reproduction	439	304	297	14
Toxicology	239	16	78	2
Aquaculture	204	109	70	11
Transcriptome	189	5	15	0
Husbandry	37	5	9	0
Total	19934	3348	2790	432

The zebrafish has preference for habitats with slow moving waters (Engeszer et al., 2007) with aquatic vegetation such as rice fields, irrigation ditches, upper reaches of rivers (McClure et al., 2006; Lawrence, 2007; Watts et al., 2012). Generally, zebrafish natural habitats are slightly alkaline (pH ≈ 8), with conductivity ranging 10 – 271 µS/cm (Engeszer et al., 2007), in shallow and high clarity waters (≈ 35 cm) (Lawrence, 2007). Zebrafish husbandry conditions in captivity mimic the optimal natural environmental characteristics, which are describe in Table 2 (Ribas and Piferrer, 2013) to promote fish welfare.

This species can achieve 1 to 5 years of lifespan in captivity, reaching 35-38 mm in adult stages (Ribas and Piferrer, 2013) with an omnivore behaviour (Ulloa et al., 2011). It is a species with sexual dimorphism (Fig. 1), males are smaller and elongated with gold and blue strips, while females have silver and blue strips, larger size and rounded abdomen (Parichy et al., 2009).

Table 2 - Zebrafish husbandry water quality parameters in captivity (adapted from Ribas and Piferrer, 2013).

	Tolerated	Ideal
Temperature (°C)	6.7 - 41.7	26 - 28
Hardness (Ca^{2+}, mg L^{-1})	75 - 200	100
Acidity (pH)	5.5 - 9	7 – 8
Ammonia (Nh_4^+, mg L^{-1})	0 - 0.05	0.002
Nitrite (NO_2^-, mg L^{-1})	0 - 0.5	0.2
Nitrate (NO_3^-, mgL^{-1})	15 - 200	50
Phosphate (PO_4^{3-}, mg L^{-1})	150 - 300	250
Dissolved oxygen (28 °C, mg L^{-1})	5.5 - 8	7.5
Conductivity (µS)	200 - 4000	750 - 1500
Alkalinity (mg L^{-1})	60 - 120	80
Copper (Cu, pg mL^{-1})	0 - 100	0 - 100
Chloride (Cl^-, mg L^{-1})	< 0-1	< 0-1

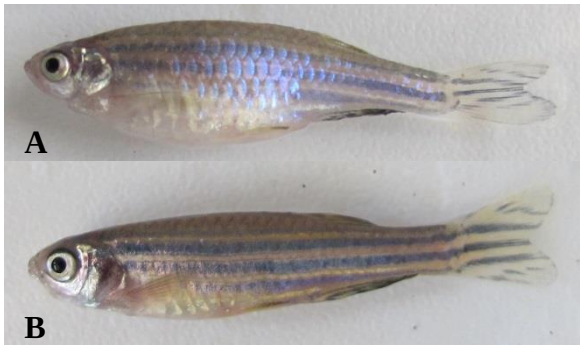


Fig. 1 - Representation of zebrafish sexual dimorphism, A - female and B – male.

Zebrafish has an important role on the identification of genes involved in the development of organs, metabolism of nutrients, response to diseases (LaPatra et al., 2000; Sanders et al., 2003; Lu et al., 2008; Rodríguez et al., 2008; Encinas et al., 2010; Cantas et al., 2012) and reproduction (Ribas and Piferrer, 2013).

Zebrafish is considered a cost-effective species in comparison with rodents (Wilson, 2012; Smith et al., 2013), since zebrafish care presents lower costs of production and maintenance, allowing an optimization of space and fish lines usage (Barut and Zon, 2000; Smith et al., 2013).

Zebrafish is studied since 1970s, but there is still an essential lack of standardization of strategies for culture and management, lacking thus rearing optimization and higher reproducibility among zebrafish facilities. Areas such as zebrafish nutrition (Jaya-Ram et al., 2008), reproduction (Sessa et al., 2008; Adatto et al., 2011; Castranova et al., 2011) and larviculture (Best et al., 2010) are still a challenge (Lawrence et al., 2012a, 2012b).

1.2 Zebrafish Nutrition

Zebrafish optimal nutritional requirements are still not completely understood, with a lack of consensus on the commercial diet usage (Siccardi III et al., 2009) and few information available related to the role of nutrition on growth and its effect in reproduction (Meinelt and Schulz, 1999; Kaushik et al., 2011). There are some studies that evaluate the effect of feeds in larval growth (Goolish et al., 1999; Carvalho et al., 2006; Kaushik et al., 2011), juvenile growth and reproductive performance (Markovich et al., 2007; Siccardi III et al., 2009; Lawrence et al., 2012a). Due to the lack of information on zebrafish nutritional requirements, larvae, juvenile and adult zebrafish are generally fed with paramecia, rotifers (*Brachionus sp.)* and artemia (*Artemia sp*.) (Watanabe et al., 1983; Westerfield, 2000; Brand et al., 2002; Lawrence, 2007; Best et al., 2010; Varga, 2011; Lawrence et al., 2012; Watts et al., 2012).

This feeding procedure does not supply a balanced nutritional diet for this species, since life feeds do not meet all zebrafish nutritional requirements. The lack, excess and bioavailability of fish essential nutrients may affect fish fitness and can promote stress (Hawkyard et al., 2011; Yossa et al., 2011). Although, live feeds are important for zebrafish rearing, since they stimulate the natural predatory behaviour of fish (Kristiansen et al., 2004; Wilson, 2012), lowering stress related to captivity, thus improving fish welfare (Cahu and Zambonino Infante, 2001). Artemia is the most practical life feed since its nauplii have the adequate size and digestibility for zebrafish larvae, and in adults it seems to improve gamete production, favourable fertilization rates and spawning performance (Meinelt and Schulz, 1999; Kaushik et al., 2011). However, artemia is costly, has intensive labour associated, unpredictable production rates and depends on the yearly natural production (Carvalho et al., 2006; Hamre et al., 2013). Moreover, the methodologies for live feeds culture are not standardized, having

a high degree of variability between laboratories, and culture facilities (Siccardi III et al., 2009; Castranova et al., 2011; Watts et al., 2012). Furthermore there are studies showing that different diets can differentially affect zebrafish gene expression. The development of an adequate and balanced zebrafish feed formulation is essential for research, since it is a species used worldwide (Watts et al., 2012).

Westerfield (2000) proposed the feeding of adult zebrafish with commercial diets and for larvae the use of fine powder commercial diets. Nowadays, numerous commercial diets appeared in the market and several formulations have been published (Siccardi III et al., 2009; Kaushik et al., 2011). Although, the composition and concentrations of these diets are unknown and may contain antinutritional compounds, such as dyes and preservative products, that may have unpredictable effects on fish fitness, or promote false positives/negatives compromising the reliability of experimental results (Siccardi III et al., 2009; Watts et al., 2012). Consequently, the development of a diet with a controlled composition is a pertinent matter to be addressed. Also, Siccardi III et al. (2009) suggested the development and common use of an open-formulation dietary standard diet for zebrafish.

The available information for zebrafish is mainly focused on the requirements in fatty acids, where growth and fertilization rates are correlated with the levels of ω-6 polyunsaturated fatty acids (PUFAs) in diet (Meinelt and Schulz, 1999; Meinelt et al., 2000). Other authors described the importance of arachidonic acid (Ara) in the reproductive performance of adult zebrafish (Meinelt and Schulz, 1999; Jaya-Ram et al., 2008; Kaushik et al., 2011) but without mention to the optimum levels of tissue-specific Ara concentrations (Gonzales and Law, 2013). There is still a lack of consensus in the zebrafish protein requirements ranging from 30 - 35 % (Ulloa et al., 2011) up to 46 - 50 % (Westerfield, 2000). This species carbohydrates demand is 35% of the diet composition (Robison et al., 2008), however the micromineral requirements have received few attention by the scientific community (Watts et al., 2012).

There are several types of diets that can be used to feed fish separated into practical diets, semipurified diets and purified diets (Table 3). Overall, the use of purified diets is the best option to study a species nutritional requirements, since they are chemically controlled and use pure ingredients, allowing the precise manipulation of nutrients (Watts et al., 2012). This type of diet can be very useful to determine zebrafish

nutritional requirements, as performed in this work for phospholipids, minerals and vitamins.

Table 3 – Classification of feeds (adapted from Watts et al., 2012).

Type	Application	Content	Cost
Purified	Experimental feed, used to evaluate macro- and micro nutrient requirements	Purified, chemically defined ingredients, usually with defined lot number and specifications	High
Semipurified	Experimental feed, used to evaluate some macro- and micro nutrient requirements	Both purified and practical ingredients, not completely defined	Moderate
Commercial	Commercial production feeds, produced in mass quantities for aquaculture operations, cannot be used to evaluate nutrient requirements	Pratical ingredients, not chermically defined	Low

1.2.1 Phospholipids

Phospholipids are essential nutrients for fish larvae normal growth and survival. The most abundant phospholipid family in biological membranes are the phosphoglycerides, due to their role structural maintenance (Tocher, 2003). The importance of the phosphoglycerides family has been reported on fish development, fish embryogenesis and early development (Koven et al., 1998; Tocher et al., 2008). In this family the most important phospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) (Tocher et al., 2008).

The pathways of *de novo* phospholipid biosynthesis are still poorly studied, however it has been shown that zebrafish has all the necessary phospholipidic biosynthesis enzymes for this process (Lykidis, 2007). In rainbow trout (*Oncorhynchus mykiss*) hepatocytes, it was demonstrated that it is possible to biosynthesise PC and PE. However this production is limited to early development stages requiring uptake by

ingestion (Coutteau et al., 1997). The PC seems to be the predominant phospholipid in fish lipoproteins, but its composition in fish plasma lipoproteins is rarely described (Tocher et al., 2008). Some studies reported PC as an enhancer of feeding activity (Koven et al., 1998) and digestion rates, that may be associated to the attractant effect of PC. This effect led to an increase of ingestion rates and improvement of larval growth and development in carp (Fontagné et al., 1998; Geurden et al., 1997, 1998; Hadas et al., 2003). This feeding attractant effect is preceded by transmembrane signalling, where PC binds to gustatory receptor cells distributed in fish body surface and the impulse produced is responsible for the stimulus to feed (Schmidt-Nielsen, 1991). Other authors reported that this phospholipid has an antioxidant role, which lowers lipid peroxidation of the intestinal tissue (Koven et al., 1998). In the sperm of fish PC is one on the major constituents of sperm membrane ranging 26 to 55% of the total constitution, reaching 80% in European sea bass (Drokin, 1993; Bell et al., 1997; Martínez-Páramo et al., 2012a). Other reports support a relationship between phospholipids and a decrease in incidence of malformations (Coutteau et al., 1997) and improved skeletal development (Cahu et al., 2003). Although, excessive levels in the diet can lead to decrease in survival and an higher rate of malformations in common carp (*Cyprinus carpio*) (Geurden et al., 1998). According to Geurden et al. (1998) PC seems to have a less effective performance in reducing skeletal deformities in comparison with other phospholipids, as the case of phosphatidylinositol (PI). A study performed in European sea bass, showed that a PI incorporated in diet at 1. 6 % of Dry Weight (DW) is important to prevent deformities during larval development (Cahu et al., 2003).

The synthesis of PE was already observed in several species, such as halibut (*Hippoglossus hippoglossus*), plaice (*Pleuronectes platessa*) and cod (*Gadus morhua*) during PC catabolism, related to the association to membrane structure and fluidity (Rainuzzo et al., 1997). In rainbow trout and European sea bass, PE is a major phospholipid in sperm composition, representing 26 and 66% in rainbow *trout* sperm (Bell et al., 1997) and 39.7 % in European sea bass (Martínez-Páramo et al., 2012a). PE presents a lower feed attractability in comparison with PC, due to the lack of a trimethyl group on their head alcohol moiety (Koven et al., 1998).

1.2.2 Minerals

Animal life processes depend on the availability of a high range of minerals for skeletal formation, regulation of organisms, hormones and enzymes (Watanabe et al., 1997). Most of the available information regarding fish mineral requirements is related to live feed (artemia and rotifers) enrichments, since it is used as first feed in fish early developmental stages (Hamre et al. 2008). Some highly relevant minerals were selected to be tested in this study such as selenium, iodine, zinc, and manganese (Table 4).

Table 4 - Classes of nutrients and sources suggested as important in zebrafish nutrition (adapted from Watts et al., 2012).

Nutrient Class	Description
Proximate nutrients	Protein (nitrogen), essential and non-essential amino acids, intact protein
	Carbohydrate (digestible), monosaccharides, oligosaccharides, polysaccharides
	Lipids, essential fatty acids, polyunsaturated fatty acids, phospholipids, cholestrol
Macrominerals	Calcium, magnesium, phosphate
Microminerals	Iron, zinc, manganese, copper, selenium, cobalt, sodium, chloride, potassium, boron
Vitamins	Ascorbic acid, tocopherols, calciferols, retinols, thiamine, riboflavin, folic acid, inositol

Selenium (SE) is an important antioxidant playing a relevant role in reproduction and immune system (Wang et al., 2013). This nutrient can be found in two forms: selenocysteine and selenomethionine. Selenocysteine is incorporated at the active site of numerous proteins (Wang et al., 2013) involved in redox reactions and acting as an essential component of the catalytic cycle (Köhrle et al., 2000). Deficient dietary selenium levels can cause severe damages on fish pancreas, as seen in Atlantic salmon (*Salmo salar*), due to decreased peroxidase activity (Bell et al., 1986). Other effects were observed in common carp, such as muscle fibre degeneration, spleen splenomegaly and congestion, liver and kidney necrosis (Wang et al., 2013) that seem to have a correlation with selenium deficiency and lipid peroxidation of diets (Lopez-Albors et al., 1995). Selenium deficiency in common carp induced growth disturbances,

increase of mortality due starvation, muscle atrophy due to myodystrophy, nutritional hepatopathy and skeletal malformations (Wang et al., 2013). An adequate dietary supplementation of SE is therefore essential for a correct fish development and welfare.

Zinc (ZN) is a mineral nutrient known to be associated with fish enzymatic activity (Nguyen et al., 2008) and normal growth (Yamaguchi, 1998; Ovesen et al., 2001; Yamaguchi and Fukagawa, 2005; Nguyen et al., 2008). Moreover, this nutrient is associated with bone formation and mineralization (Ovesen et al., 2001; Nguyen et al., 2008), through activation of aminoacyl-tRNA synthetase in osteoblastic cells that stimulates cellular protein synthesis (Nguyen et al., 2008). During embryonic development, ZN plays an important role as a regulator of cell division and morphogenesis, as well as an inducer of cell differentiation in zebrafish gills (Zheng et al., 2010). The ZN content in zebrafish embryos varies considerably during cleavage, decreasing slowly during gastrula stage, mainly due to the capability of metal exchange with the environment (Riggio et al., 2003). The deficiency in ZN leads to a deficient cell division, congenital malformations and growth retardation in rainbow trout, mainly due to the reduced carboxypeptidase activity that decreases the digestibility of protein and carbohydrates (Watanabe et al., 1997; Nguyen et al., 2008). High levels of ZN have toxic effects on biological systems (Riggio et al., 2003). Consequently a balanced input of zinc for species specific requirements is essential for optimal development and reducing structural deformities (Nguyen et al., 2008).

Manganese (MN) is known to be involved in bone-organic matrix development and it is correlated with stress tolerance in fish. This micronutrient is an important co–factor for the function of a large number of enzymes and forms of metal-enzymes complexes (Nguyen et al., 2008). MN is also one of the constituents of metalloproteins found in carbohydrates and lipids (Watanabe et al., 1997; Nguyen et al., 2008). The deficiency in this mineral lead to shortened and thickened bones and for growth retardation, dwarfism and cataracts in common carp and rainbow trout (Nguyen et al., 2008). These effects are a result of a reduction in copper-zinc superoxide dismutase and manganese superoxide dismutase activities in cardiac muscle and liver (Watanabe et al., 1997). In addition, a reduction in MN content influences bone composition and disturbs bone formation (Nguyen et al., 2008). The nutritional requirement of MN (12 - 42.8 $\mu g\ g^{-1}$ DW) is normally associated to growth improvement, but other factors can synergistically be associated to its role.

Another important nutrient for fish health is iodine (I) (Hawkyard et al., 2011) since it is a precursor of thyroid hormones (THs) (Power et al., 2008), and is involved in larval growth increment and survival (Hawkyard et al., 2011; Ribeiro et al., 2012). I is essential for maintenance of THs homeostasis, where a dysfunction can lead to deregulation of cellular metabolism and abnormal development (Penglase et al., 2013). However, excessive amounts of iodine affect normal thyroid hormone production, known as the Wolf-Chaikoff phenomenon (Wolff and Chaikoff, 1948). In zebrafish inhibition of THs reduced fin and scale development, and produced pigmentation deficiencies (Brown, 1997). According to Venturi and Venturi (1999), I has an important antioxidant potential in scavenging reactive oxygen species (ROS) that are required as co-substrate of thyroid hormone synthesis. A decrease in I imply a higher H_2O_2 generation leading to cell damages (Maier et al., 2007). Nevertheless, further studies are needed to evaluate the antioxidant function of I in fish larvae (Hawkyard et al., 2011).

1.2.3 Vitamin D

Vitamins are organic compounds required in small amounts, that are essential for fish development, growth, survival, health, reproduction and maintenance (Lock et al., 2010; Yossa et al., 2011). Vitamin D is important for endocrine system and regulation of calcium and phosphate metabolism, as a regulator of transcellular calcium uptake and for inducing cytosolic calcium transport (Bronner, 2009). This vitamin is also associated to bone formation and remodelling (Lall and Lewis-McCrea, 2007; Lock et al., 2010), osteoblast activity and osteoclast formation, playing an important role in controlling growth and normal tissues (Anderson and Atkins, 2008; Samuel and Sitrin, 2008). Furthermore, vitamin D is related to cell proliferation and differentiation (Samuel and Sitrin, 2008), and its deficiency may cause gastrointestinal disorders, cardiovascular diseases and bone diseases such as osteoporosis (Grant, 2006; Bikle, 2007). Vitamin D3 is the primary storage form of vitamin D and has a higher bioavailability in fish, increasing their nutritional uptake from the diet (Lock et al., 2010). Consequently, these functions are essential to maintain fish health and a proper skeleton mineralization (Anderson and Atkins, 2008). The injection of calcitriol (1, 25 – $(OH)_2D_3$), the active form of vitamin D, induced demineralization of bone in tilapia (Lall and Lewis-McCrea,

2007). However in Atlantic salmon no effects were observed in fish treated with D3 (Graff et al., 2002). Calcitriol also seems to stimulate osteoblastic secretion of osteocalcin that is involved in the bone formation process (Lall and Lewis-McCrea, 2007). According to Lock et al. (2010) vitamin D3 (D3) metabolites interaction with bone seems to be complex, with other factors involved in the process. The combination of vitamins and their quantitative requirements are responsible for changes in the physiological and reproductive status of the fish (Watts et al., 2012). Thus, the study of vitamins and their effects are essential to understand and enhance their role in fish growth and reproduction.

1.3 Reproduction

Zebrafish is a model species that has been recently used to understand the processes involved in regulation of reproduction (Ribas and Piferrer, 2013), however, this species still needs a more thorough characterization of its gametes (Wilson-Leedy et al., 2009). The reproduction control is still one of the main bottlenecks in this species production, where a more solid husbandry knowledge is required (Ribas and Piferrer, 2013). Some advances have been made regarding the study of gonad development (Rodríguez-Marí et al., 2010), courtship behaviour (Nasiadka and Clark, 2012), egg and sperm quality (Wilson-Leedy et al., 2009), as well as germplasm cryopreservation (Robles et al., 2009; Reinardy et al., 2013) leading to a better understanding of zebrafish reproduction.

Zebrafish sex determination is still poorly understood, however it is known that it is triggered by a complex interaction of genetic (Matsuda et al., 2002; Tong et al., 2010) and environmental factors (Tong et al., 2010). At 40-42 dpf zebrafish sex differentiation is complete and at 60 dpf reproductive cells reach full maturation (Baroiller et al., 2009).

Furthermore, zebrafish can be sex reversed by exposure to estrogens (von Hofsten and Olsson, 2005) and can have natural sex reversal along their lives (Uchida et al., 2002).

It is essential a full understanding of zebrafish reproductive mechanisms to improve one of the main production bottlenecks and to obtain high quality gametes and high quality progeny (Wilson-Leedy and Ingermann, 2007).

1.3.1 Gametes quality

The control of reproduction is a key issue for fish production being the quality and availability of the female and males gametes a limiting factor (Bobe and Labbé, 2010). The use of high quality broodstock is priority to ensure high quality gametes increasing the fertilization success. Several factors such as stress in captivity, water quality, diseases, nutritional disorders and husbandry management conditions affect the broodstock decreasing gametes quality (Migaud et al., 2013). High quality gametes are essential to achieve high fertilization rates, but also to develop healthy larvae development thorough good egg nutritional reserves and both gametes genetic integrity (Rurangwa et al., 2004). Zebrafish as most teleosts have external fertilization (Cabrita et al., 2009). When the sperm is released the motility is triggered by hypoosmotic schock and ionic exchanges of Ca^{2+} with the environment leading to sperm activation (Takai and Morisawa, 1995; Wilson-Leedy et al., 2009). Zebrafish sperm motility lasts 60 seconds, during this period sperm penetrates the micropyle before it closes, and exhaustion of mitochondrial ATP reserves, and ensure the reproduction success (Rurangwa et al., 2004; Diogo et al., 2010). The sperm release consists in a critical period in fertilization since freshwater is an extremely hazardous medium for sperm sensitive cells (Diogo et al., 2010), consequently the evaluation of gamete quality is essential in order to evaluate the success of reproduction (Cabrita et al., 2009; Diogo et al., 2010).

The inexistence of an egg quality universal marker, led to the use of several methodologies to evaluate egg quality, such as determining egg diameter, embryo diameter and perivitelline space. The diameter and perivitelline space are associated to a good larvae development (Bobe and Labbé, 2010), due to the amount of yolk reserves in the eggs (Cabrita et al., 2009). Other tools have been used to evaluate the egg quality, such as the blastomere morphology (Lahnsteiner et al., 2002; Kjørsvik et al., 2003), ovarian fluid composition (Lahnsteiner et al., 1999; Diogo et al., 2010), biochemical composition of egg and molecular analysis of genes expression (Cabrita et al., 2009). Egg quality is associated to incorporation, synthesis, and processing of egg components during oogenesis that will be of extreme importance for the adequate larval development (Bobe and Labbé, 2010; Nasiadka and Clark, 2012). According to Izquierdo et al. (2001) the broodstock nutrition can have significant impacts in reproductive performance and embryo development. The nutritional disorders can cause

effects in spawn quantity and frequency of female and male gametes (Bobe and Labbé, 2010). A study performed in Atlantic salmon suggests that the lipid source during gonadal maturation can affect egg fatty acid composition (Pickova et al., 1999). The quality of both paternal and maternal gametes contribute to success of reproduction and larval viability (Kamler, 2005). The gamete quality is important for reproduction success, although sperm can contain small DNA damage which could be repaired by egg repair mechanisms (Cabrita et al., 2009).

Sperm quality is by definition the ability to successfully fertilize an egg in an appropriate environment and produce viable embryos (Rurangwa et al., 2004; Cabrita et al., 2009). Sperm condition can be affected by several factors as genetic, physiological or environmental cues and their complex interactions that can be responsible for the decrease in the ability to fertilize, compromising the reproduction success (Rurangwa et al., 2004).

There is a lack of consensus in sperm quality evaluation indicators, since the quality cannot be evaluated based in a particular sperm characteristic but on its overall fitness. The sperm motility is the general criteria used for sperm quality evaluation and has been used an indicator of sperm quality. This analysis allowed the observation of effects of treatments and probability of fertilization success and/or the interaction between both factors (Cabrita et al., 2009). Also, sperm motility has been used to evaluate the effect of nutritional disorders, stress, husbandry and environmental conditions. Computerized assisted tools as CASA (Computer Assisted Sperm Analysis software) are valuable tools used to perform sperm motility analysis (movement, trajectory and velocity), through video recording of sperm movement (Bobe and Labbé, 2010). This software quantifies motility through analysis of several parameters for each spermatozoon such as, percentage of total motile sperm (TM), progressive spermatozoa (PM), linearity (LIN), curvilinear velocity (VCL) and straight line velocity (VSL). These parameters described by Boyers et al. (1989) allow an accurate evaluation of sperm, also providing information regarding spermatozoa physiology (Cabrita et al., 2009). The analysis of sperm motility is considered a good index for sperm quality analysis, allowing the improvement of protocols for several species, as the case of zebrafish, since each species present a different sperm physiology of movement (Wilson-Leedy and Ingermann, 2007; Cabrita et al., 2009).

1.4 Skeletal Deformities

The onset of deformities in several marine species frequently occurs during larval development. The presence of deformities is slightly reduced (5%) in the juvenile and adult stages (Andrades et al., 1996; Sandel et al., 2010; Boglione et al., 2013b). Other authors pointed the presence of deformity values close do 90% of total larvae production (Boglione et al., 2013b). According to Boglione et al. (2013b) a value close to 20% of deformities at the end of hatchery phase is considered good, but rarely achieved.

The presence of deformities in fish skeletogenesis was reported for the first time in 1883 in *Syngnathus Peckianus* (McMurrich, 1883). The Rainbow trout is one of the first modern domesticated fish species and was the first produced species with reports on bone development and deformities (Aulstad and Kittelsen, 1971). Nowadays, occurrence of skeletal anomalies has been reported in several species such as Atlantic salmon (Gil-Martens, 2010), European sea bass, gilthead sea bream (*Sparus aurata*) (Barahona-Fernandes, 1978), ayu (*Plecoglossus altivelis*) (Komada, 1980), senegalense sole (*Solea senegalensis*) (Gavaia et al., 2002, 2009) and Atlantic halibut (Lewis and Lall, 2006; Lewis-McCrea and Lall, 2010) among others. Some of the produced species present values close to 90% or up to 100% of deformities (Boglione et al., 2001; Gavaia et al., 2009). Fish produced in captivity present an higher incidence of deformities compared to the natural habitat, mainly due to the lack of natural selection and problems related to unsuitable fish rearing protocols (Gavaia et al., 2009).

Skeletal deformities can be inherited (McKay and Gjerde, 1986; Bonnet et al., 2007a, 2007b) caused by environmental pathologic, zootechnical aspects (Chatain, 1994; Andrades et al., 1996; Divanach et al., 1997) or dietary factors (Cahu et al., 2003). In reared species, inappropriate feed formulations in unsaturated fatty acids requirements, unbalanced vitamins A, C, D and K (Udagawa, 2001; Cahu et al., 2003; Lall and Lewis-McCrea, 2007; Fernández et al., 2008) and their hypervitaminosis (Fernández et al., 2011) are responsible for the presence of deformities (Andrades et al., 1996; Afonso et al., 2000; Cahu et al., 2003; Izquierdo et al., 2010; Koumoundouros, 2010; Boglione et al., 2013b). In reared species, the most common deformities affecting the vertebral column are lordosis, kyphosis, scoliosis, and fusions in vertebrae, haemal and neural arches (Boglione et al., 2013a). There are several ethical issues involved in deformities,

since deformed mouth, fins and vertebral axis, affect the feeding capacity and swimming ability interfering in fish welfare. These deformities lead to lower feeding rates, slower growth rates and stress and pathogenic susceptibility (Andrades et al., 1996; Koumoundouros et al., 1997; Boglione et al., 2001, 2013a). The identification of the causative effects of deformities is difficult to be assessed due to the synergistic effect between abiotic and non-abiotic factors (Boglione et al., 2013a). Several diagnostic tools have been used to evaluate the deformities incidence, such as external observation, X-rays, palpation, staining, synchrotron microcomputer tomography, computed tomography, histopathology, histochemistry, immunohistochemistry, depending on life stages of development (Boglione et al., 2013b).

Little information can be found regarding wild type zebrafish deformities, with the available information related to caudal fin fusions as the most affected region (Bensimon-Brito et al., 2010). The remaining information is referent to mutant lines (e.g. *din* mutants) and the effects in axial skeleton (Fisher and Halpern, 1999). The presence of deformities in zebrafish regarding nutritional requirements studies are still scarce. The information available is mainly related to probiotic- supplementation on diet, with observation of faster development with earlier onset of backbone calcification (Avella et al., 2012; Carnevali et al., 2013).

1.5 Zebrafish bone mineralization

The understanding of teleost fish bone remodelling processes is essentially important for different areas as aquaculture (skeletal malformations) and biomedical research (skeletal development) (Witten and Huysseune, 2009). Most teleosts present an acellular bone (lacks osteocytes) which allow the continue development throughout life (Boglione et al., 2001; Witten and Huysseune, 2009). Acellular bone present characteristics such as absence of osteocytes, absence of haematopoietic bone marrow, abundance of small mononucleated osteoclasts, phosphorus mineral homeostasis and skeletal resorption often absent form particular sites (Witten and Huysseune, 2009). The zebrafish has a different bone type, with cellular bone, a conformation more similar to mammals (Parenti, 1986). In zebrafish, bone remodelling is characterized by resorption by osteoclasts (either mono- or multinucleated osteoclasts) and subsequent formation of new bone by osteoblasts (Witten et al., 2001; Witten and Huysseune, 2009). This

characteristics makes zebrafish an ideal model to study bone development and bone diseases (Witten et al., 2001). During the last decades, zebrafish has been studied as a model for vertebrate bone development, with focus on cellular and genetic aspects of bone and scale formation (Witten and Huysseune, 2009).

Zebrafish axial skeleton was described by Bird and Mabee (2003) as composed by 31 vertebrae (including the urostyle), which are divided into 4 weberian vertebra, 10 pre-caudal vertebra (bearing neural arches and spines, parapophyses, and ribs) and 17 caudal vertebra (bearing neural and haemal arches and spines) including 3 caudal fin vertebra modified in order to support the caudal fin (Fig. 2).

The zebrafish skeletal development was described by Gavaia et al. (2006), where the first stages of development (48-52 hpf – hours post fertilization), only present a underdeveloped skeleton composed by cartilaginous elements, without presence of calcified structures. The presence of mineralized structures just started to be noticed after 96 hpf, when the mineralization of basioccipital articulatory process (BOP) occurred, representing the first axial skeleton structure to be mineralized. The mineralization of vertebra over the notochord started after the larvae were able to feed, at 9 dpf (days post fertilization), where the mineralization occurred from the dorsal to the ventral side of the vertebra. The five hypurals, parhypural, and modified haemal arches, constituents of the internal skeleton of the caudal fin were already mineralizing at 12 dpf, and the first calcified rays of the caudal fin formed by intra-membranous calcification. In relation to the neural arches, the first calcification was seen at 14 dpf. The formation of the vertebral column was completed at 19 dpf, where all the structures were already calcified, with exception of the two most posterior vertebrae that form the urostile.

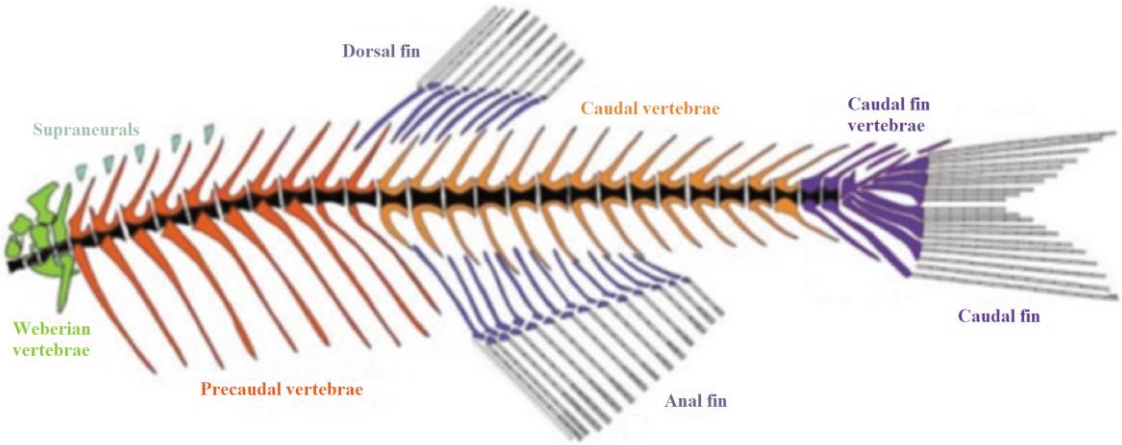


Fig. 2 - Generalized diagram of the zebrafish axial skeleton. Centra are in black, the Weberian apparatus is green, supraneurals are light blue, precaudal vertebrae are red, caudal vertebrae are orange, the caudal fin skeleton is purple, and the dorsal and anal fin endoskeletons are blue (adapted from Bird and Mabee, 2003).

OBJECTIVES

2. Objectives

The aim of this study was to evaluate the effect of nutritional modulation in vitamins, phospholipids and trace minerals using a purified diet in adult zebrafish growth and reproductive success and subsequently in the larval growth, survival and skeletal development.

MATERIAL AND METHODS

3. Material and Methods

3.1 Rearing conditions

The experimental work was performed in the laboratories and zebrafish rearing facilities of the Centre of marine sciences (CCMAR) at the University of Algarve. Adult zebrafish were maintained in a 14:10 light-dark photoperiod cycle, housed in a ZEBTECH (Tecniplast, Italy) recirculating system with 3L glass fish tanks with 7,3 L h^{-1} water turnover. The water was previously treated by a reverse osmosis system (Aquatic biosystems, UK) and maintained at 28 ± 1 ºC, 680 µS of conductivity and a pH of 7.5. The nitrite, nitrate and ammonia levels were monitored weekly, being constantly within the accepted limits throughout the experiment (< 0.1mg L^{-1}).

3.2 Nutrition

To analyze the effect of a control diet (CD), a purified diet (PURE) and 7 supplemented purified diets (constituting 9 treatments in total), a group of 8 fish per treatment (5 females and 3 males), with 3 months of age, were selected from wild type stock and fed for 20 days with each diet before starting the reproduction trials. A high quality commercial marine fish larval diet (CD, 60% Crude protein; 14% Crude Lipid) and a purified diet (Table 5) were used as controls. On the remaining diets used, the purified diet was supplemented with: phosphatidylcholine (PC, 5 g/Kg), phosphatidylethanolamine (PE, 5 g/Kg), selenium (SE, sodium selenite, 1 mg/Kg), zinc (ZN, zinc oxide, 750 mg/kg), manganese (MN, manganese oxide, 1 mg/Kg), iodine (I, potassium iodate, 20 mg/kg) and vitamin D3 (D3, cholecalciferol, 4000 UI/Kg). The adult fish were fed with pellets ranging 600-800 µm in size and the larvae with a fraction ≤ 200 µm. Each experimental diet was offered 2 times a day at *ad libitum* regime and all tanks were siphoned for removing debris approximately 30 minutes after each feeding.

Larvae were fed from 5 to 7 days post fertilization (dpf) in co-feeding, with two meals of 200 µm diets and one intercalary meal of artemia AF nauplii (5 nauplii ml^{-1}; INVE Tech., Dendermonde, Belgium). Once the co-feeding period was finished the larvae

were only fed with treatment diets until the end of the experiment. To hatch artemia nauplii, 1g L^{-1} of AF cysts was incubated in seawater at 35 ppt salinity, 28 °C temperature and with a strong aeration for 24 hours (Sorgeloos et al., 2001). The newly hatched artemia was collected and rinsed on a 150 µm sieve with freshwater to eliminate bacteria and diluted in system water for feeding to avoid osmotic stress to fish.

Table 5 – Purified diet (PURE) composition (%) with respective proximate composition used during the experimental period.

Ingredient, %	PURE
Fish gelatina	16
Casein	40
Wheat glúten	5
Dextrine	14,3
Cellulose	4,1
Fish oil	11,5
Vit & Min Premix PV01	2
Betaine	0,1
Soy lecithin	0,2
Binder (sodium alginate)	2
Antioxidant	0,2
MCP	2,7
L-Lysine	1
DL-Methionine	0,5
L-Arginine	0,4
proximate composition	
Crude protein (g Kg^{-1} DM)	60,4
Digestible protein (g Kg^{-1} DM)	56,2
Crude fat (gKkg^{-1} DM)	12,1
Fiber (g Kg^{-1} DM)	0,0
Starch (g Kg^{-1} DM)	14,5
Gross Energy (Kj g^{-1} DM)	21,2

3.2 Growth performance

The broodstock was sampled for weight, standard length (SL) and total length (TL), at the beginning, middle and end of the experiment (25 - 68 days). Growth performance indicators were analyzed through calculation of Weight gain (IBW, %), Daily growth

index (DGI, %), Average body weight (ABW, g), Condition factor (CF, %) and Specific growth rate (SGR, mg/g) indexes (Fig. 3).

A - Weight gain (IBW, %)

$$= 100 \times \frac{\text{final body weight (g)} \ - \ \text{initial body weight(g)}}{\text{initial body weight (g)}}$$

B - Daily growth Index (DGI, %)

$$= 100 \times \frac{(\text{final body weight})^{\frac{1}{3}} - (\text{initial body weight})^{\frac{1}{3}}}{days}$$

C - Average body weight (ABW, g)

$$= \frac{(final\ body\ weight + initial\ body\ weight)}{2}$$

D - Condition factor (CF, %)

$$= 100 \times \frac{final\ body\ weight\ (g)}{final\ lenght\ (cm)}$$

E – Specific growth rate (SGR, mg/d)

$$= 100 \times \frac{(Ln\ (final\ body\ weight) - Ln\ (initial\ body\ weight))}{T\ (days)}$$

Fig. 3 - Growth indexes for adult zebrafish (adapted from Gonzales and Law, 2013 and Silva et al., 2010).

The specimens from the different broodstock groups were photographed individually with a digital camera (Canon Powershot G12; Canon, Japan) and measured using imaging software for image analysis Axio Vision (Carl Zeiss, Germany) to determine total length (TL) and standard length (SL). The samples were properly stored at -80°C, for further analysis of chemical composition.

To analyze larval total and standard length 10 larval specimens were collected at 5 dpf, 8 dpf, 15 dpf and 28 dpf. The fish were properly anesthetized with MS-222 (Sigma-

Aldrich, Saint Louis, MO, USA) and photographed for length measurements (TL and SL) as described above.

3.3 Broodstock reproduction

Fish from both sexes from each broodstock group were kept separately, allowing controlled reproduction and high gamete quality. Once a week, fish from each group were mated to ensure a washout of eggs matured in pre-feeding conditions. Fish were placed on 3 L glass spawning tanks during the evening. Males and females were maintained separated by nets overnight. In the following morning the separation was removed and fish were allowed to get together for mating in low water depth. After 6 weeks of experimental feeding and mating, zebrafish males from each treatment were sampled for sperm motility analysis.

3.4 Sperm mobility collection and analysis

To analyze sperm quality, a motility analysis was performed at the end of the trial. For that, the males from each treatment were anesthetized with MS-222 and the sperm collection was performed through abdominal massage by gently pressing abdomen region with wide flat straight forceps. The urogenital pore was carefully cleaned and dried before the sperm collection. The samples were diluted in 10 µl of system water for activation of spermatozoa motility and immediately placed on a Makler chamber and analyzed at 15, 30, 45 and 60 seconds (s) post activation. The activation was performed in 1.5 ml microtubes at 7 ºC to avoid sperm quality degradation. The spermatozoa motility analysis was performed using the Computer Assisted Sperm Analysis software - CASA (ISAS, Proiser, Valencia, Spain). A computer with the software control was connected to a digital recording camera (Basler 312f, Basler Afc, Germany) attached to an optical phase-contrast microscope (Nikon E200, Tokyo, Japan). For this experiment the spermatozoa motility parameters considered more adequate for fish were selected as follows: total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s) and linearity of the spermatozoa path

(LIN, %) (Boyers et al., 1989; Migaud et al., 2013). Data was recorded at 15, 30, 45 and 60s.

3.5 Sampling of eggs

The newly fertilized eggs were collected and immediately bleached, to avoid pathogenic contaminations, as described by Westerfield (2000). A sample of 10 eggs from each treatment was collected and photographed with a Canon Powershot G12 digital camera coupled to a stereomicroscope (Leica MZ6; LeicaMicrosystems, Wetzlar, Germany) and measurements of perivitelline space and total egg diameter were recorded using Axio Vision and were used to evaluate egg quality.

3.6 Larvae rearing

The eggs were incubated in 1 L reproduction tanks (Tecniplast) at 28 °C, without water recirculation, with methylene blue added at 0.01% (approximately 50 µl L^{-1}) as described by Brand et al. (2002) in order to reduce bacterial and fungal growth. The water of each tank was completely exchanged every 2 days. The hatching rate was evaluated and the mortality was daily monitored. The larvae were reared in 1 L tanks until the end of the experiment at constant temperature of 28 ºC and a 14:10 h photoperiod.

To analyze weight, total and standard length, 10 larvae from each treatment were collected at 5 dpf (days post fertilization), 15 dpf and 28 dpf. The fish were properly anesthetized with MS-222 and photographed with a Canon Powershot G12 digital camera. The length measurements (TL and SL) were performed with Axio Vision software.

3.7 Mineralization analysis

To evaluate larvae performance and mineralization conditions, the larvae were euthanized with a lethal dose of MS-222 and dehydrated at 60°C during 24h.

To perform bone mineralization analysis the samples were incinerated in a furnace at 600 °C during 12 hours in a previously desiccated and weight calibrated ceramic melting pot. The ashes were then weighted and re-suspended in a 500 µL of HCl 6 N solution. The suspension was used to analyze the larval mineralization levels by using the QuantiChrom^{TM} Phosphate Assay Kit (DIPI-500; BioAssay Systems, CA, USA) according to the manufacturer's specifications.

3.8 Histological procedures

The larvae at 28 dpf were anesthetized with a lethal dose of MS-222 and fixed in a 4% buffered paraformaldehyde solution at 4°C for 24 hours before entering the whole-mount bone and cartilage double staining procedure according to the described in Walker and Kimmel (2007).

Larvae were washed with a 0.1 M phosphate buffer saline (PBS), pH 7.4 solution and stored in ethanol 75% during 1.5 hours at room temperature. The acid-free double staining protocol was performed with an alcian blue 8GX solution for cartilage detection and alizarin red S for bone detection. The samples were stained with alcian blue 8GX for 1.5 hours and posteriorly passed in a decreasing series of ethanol concentrations (96 - 25%), and hydrated with distilled water before being stained with alizarin red S in a potassium hydroxide solution 1% overnight (adapted from Walker and Kimmel, 2007; Gavaia et al. 2000).

The soft tissues were cleared with a potassium hydroxide solution at 0.5% for 2-3 days. Samples were stored in an aqueous solution of 50% glycerol at room temperature until being analyzed. Some samples were bleached with a 10 µL/ml hydrogen peroxide at 30 vol. solution to help remove pigmentation.

3.9 Statistical analysis

The data was normalized, with arcsen, and the adequate statistical analysis was performed. The data was subjected to a One-way ANOVA ($p \leq 0.05$) analysis of variance for hatching rate, number of deformities, charge of deformities and presence of deformities by area. The data related to Breeders length (SL and TL), Breeders weight,

Sperm motility analysis (TM, PM, LIN, VCL and VSL), and larvae length (SL and TL) were analyzed by a repeated measures ANOVA ($p \leq 0.05$). In all the data a Tukey´s multiple range test ($p \leq 0.05$) were performed to compare averages. All the statistical analysis was performed in (SPSS 21.0).

RESULTS

4. Results

4.1 Growth in length and weight

The different groups of adult zebrafish submitted to different supplemented diets were sampled in 3 different points to determine the effect of the different diets in growth by measuring length (TL and SL) and weight as biological performance markers. At 25 days of experiment (M), the control commercial diet (CD; 35, 9 mm) breeders presented the largest sizes with significant difference ($p = 0.002$) in standard length (SL) in comparison with D3 diet (D3; 28, 9 mm) (Fig. 4a). At the end of the experiment (E; 68 days), zebrafish fed with CD presented better results in terms of growth in SL ($p = 0.002$) than those fed with PE, ZN, MN, I and D3 diets. The poorest results at all sampling points were observed for the D3 diet (Fig. 4a).

Regarding total length (TL), at 28 days of experiment (M), CD and PC diets showed a significantly larger length ($p = 0.002$) in comparison with the D3 diet that presented the lower values (Fig 4b). At the end of the experiment (E) the CD and PC groups had the better values ($p = 0.0001$) for TL, in comparison with those fed with PE, MN, I and D3 diets. The control purified diet (PURE) and the SE diet also exhibited significantly different values than zebrafish fed with D3 diet (Fig. 4b).

The adult zebrafish weight showed results similar to the observed for length (Fig. 5). At 25 days (M) the CD diet presented the largest weight with significant differences ($p = 0.001$) to the ZN, I and D3 diets. Significant differences were also observed between the PC diet and those fed D3 diet. At 68 days (E) the CD diet (0. 60 mg) had the larger increment in weight in comparison with the D3 diet (0. 28 mg). No statistical differences were observed between CD and D3 and those fed the other diets (Fig. 5).

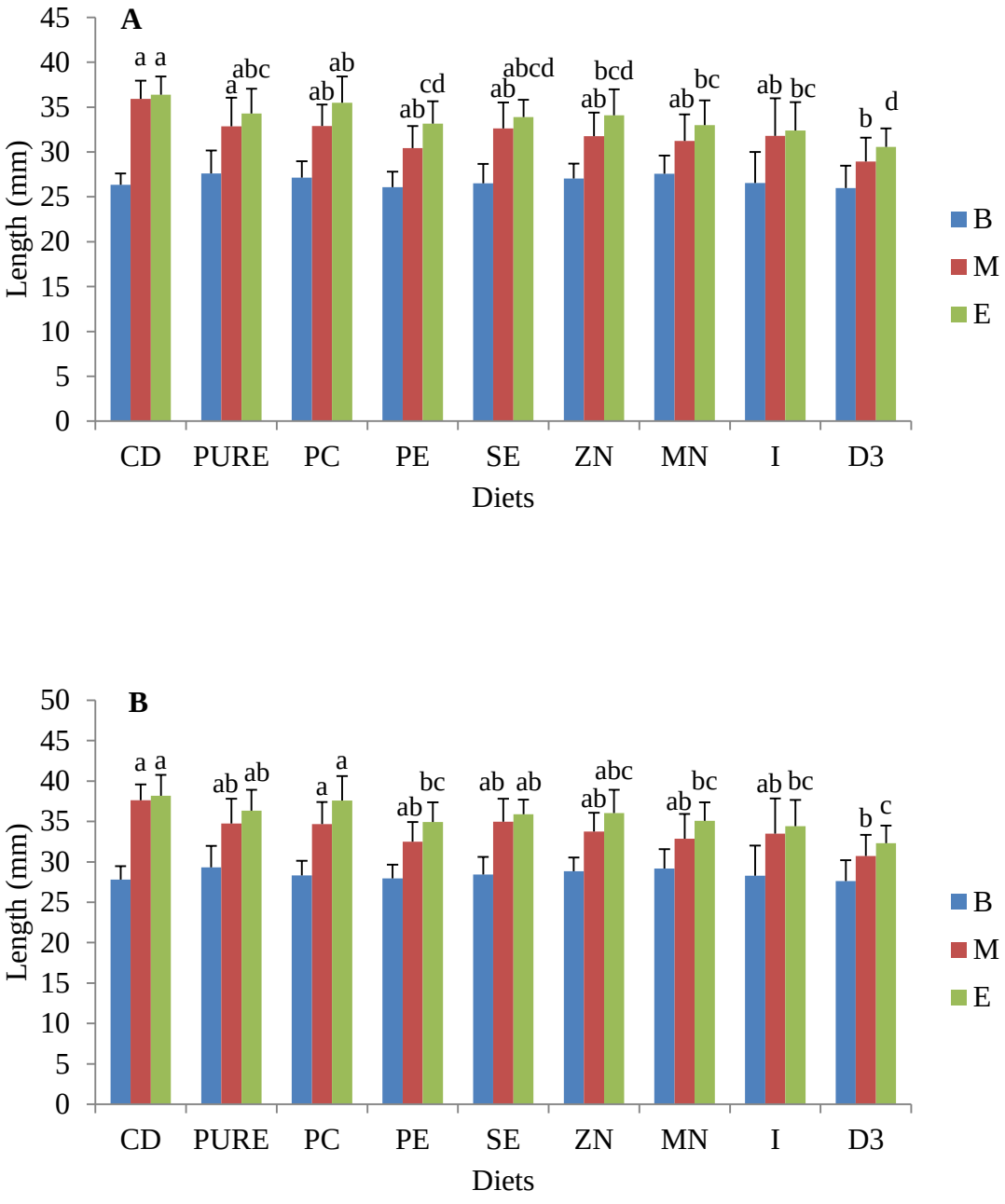


Fig. 4 – Effect of commercial diet (CD), purified diet (PURE) and supplemented diets (PC, PE, SE, ZN, MN, I , D3) in zebrafish (*Danio rerio*) breeders length (mm) Measurements at the beginning (B; 0 days), middle (M; 25 days) and end (E; 68 days) of experiment. A - standard length (SL) and B - total length (TL). Statistical differences ($p < 0.05$) are represented with letters.

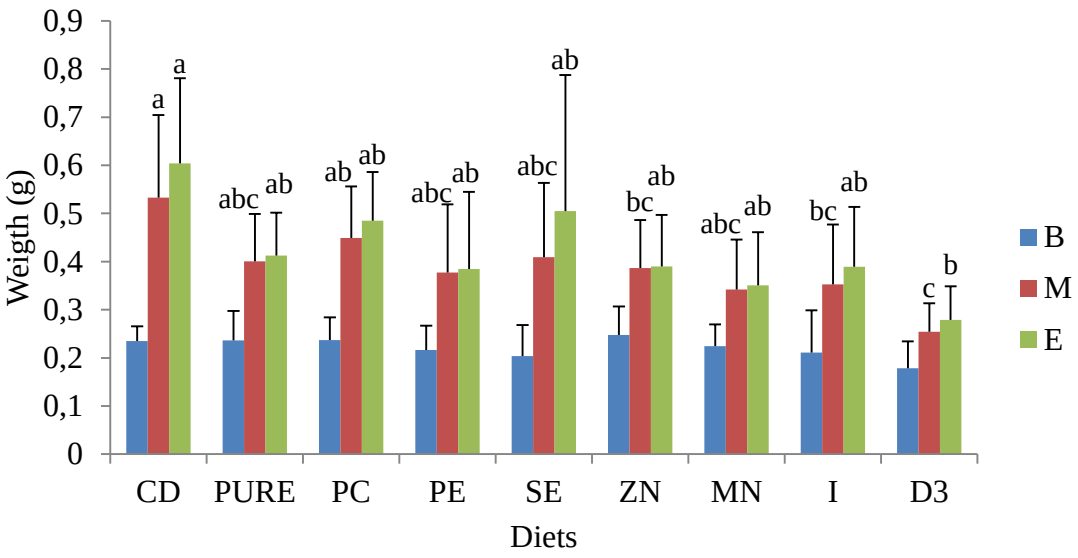
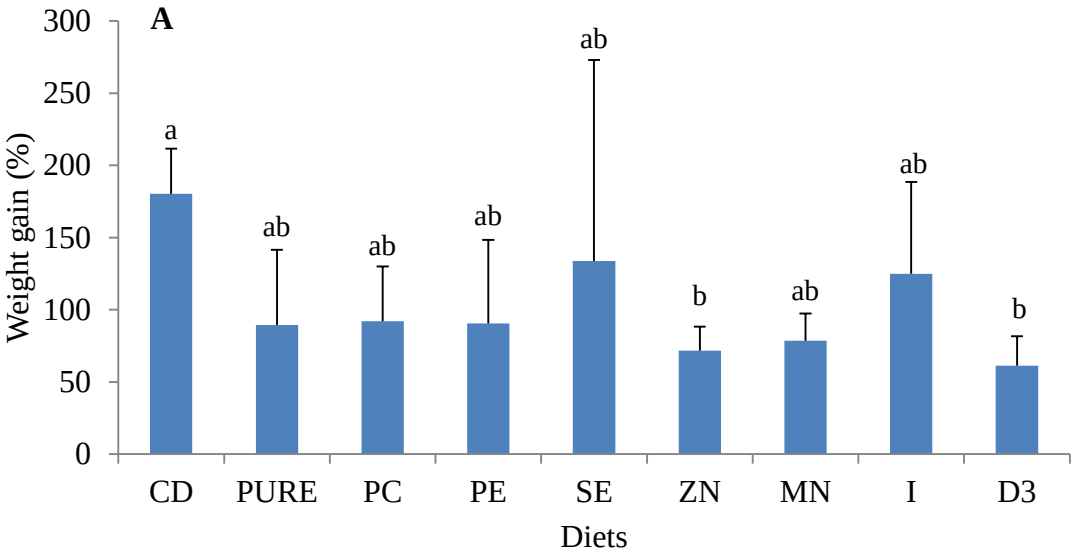
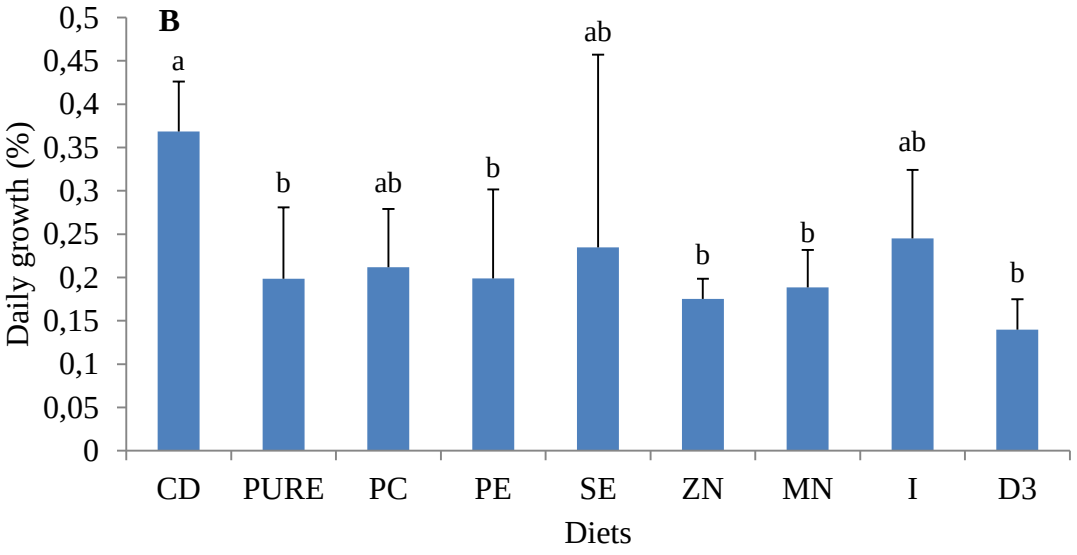


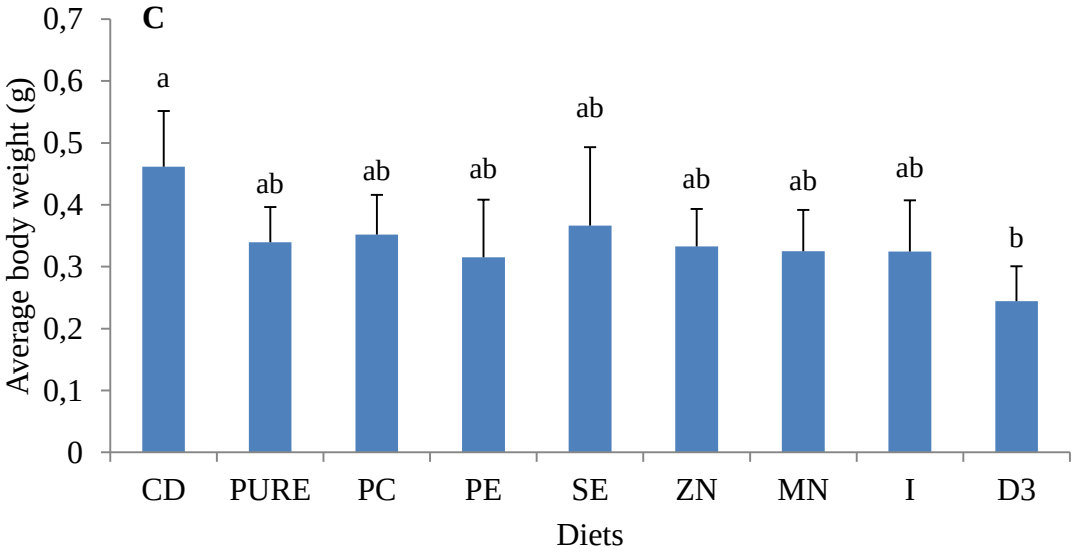
Fig. 5 – Effect of control diet (CD), purified diet (PURE) and supplemented diets (PC, PE, SE, ZN, MN, I, D3) in zebrafish (*Danio rerio*) breeders weight (g). Values recorded at the beginning (B), middle (M; 25 days) and end (E; 68 days) of experiment. Statistical differences ($p < 0.05$) are represented with letters.

4.2 Growth analysis

In addition to size and weight analysis, several indexes were used in order to ensure a better understanding of the diet effects (Fig. 6). Specimens from the control diet (CD) (180, 3%) presented a positive difference ($p = 0.026$) of weight gain (IBW) in comparison with those fed with ZN (71, 6%) and D3 (61, 3%) diets (Fig. 6a). No statistical differences ($p = 0.010$) were observed between zebrafish fed with the other dietary treatments (Fig. 6a). The CD diet had the highest ($p = 0.011$) daily growth (DGI) in comparison with PUR, PE, ZN, MN and D3 dietary treatments (Fig 6b). The diets PC, SE and I diets did not present differences in comparison with the other diets (Fig 6b). The average body weight ($p = 0.010$; ABW) and condition factor ($p = 0.050$; CD) indexes presented identical results, where the highest values were observed in fish fed CD in comparison with those fed with D3 diet (Fig. 6c, d). No significant differences were obtained relatively to the remaining diets (Fig 6c, d). Regarding the specific growth rate (SGR), the CD diet had the highest growth values ($p = 0.044$) significantly different from the ZN and D3 diets that showed the lowest growth (Fig. 6e). No differences were observed relatively to the other treatments (Fig. 6e).


A
Weight gain (%)
300
250
200
150
100
50
0
a
ab
ab
ab
ab
b
ab
ab
b
CD
PURE
PC
PE
SE
ZN
MN
I
D3
Diets


B
Daily growth (%)
0,5
0,45
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
a
b
ab
b
ab
b
b
ab
b
CD
PURE
PC
PE
SE
ZN
MN
I
D3
Diets


C
Average body weight (g)
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
a
ab
ab
ab
ab
ab
ab
ab
b
CD
PURE
PC
PE
SE
ZN
MN
I
D3
Diets

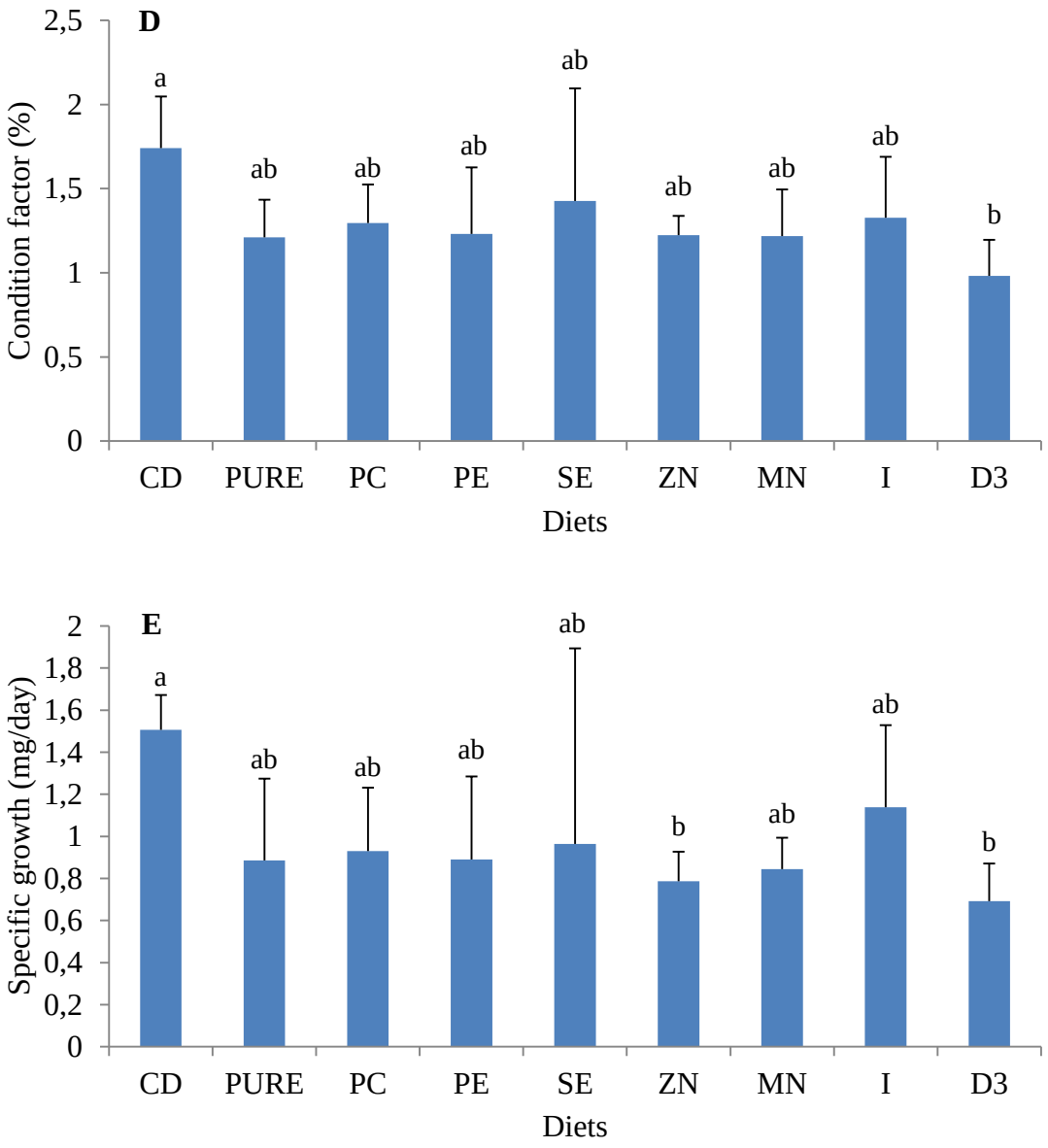
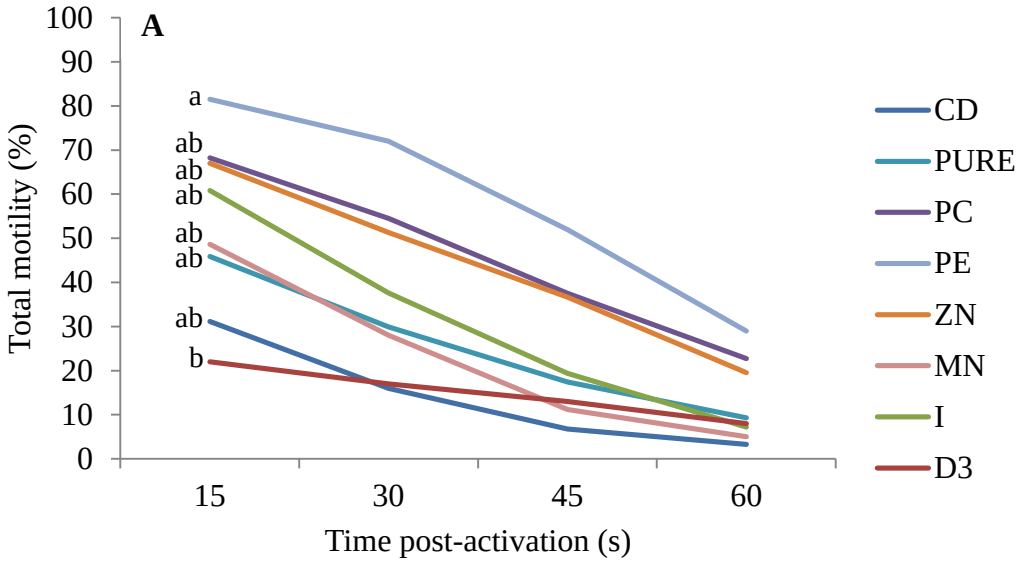


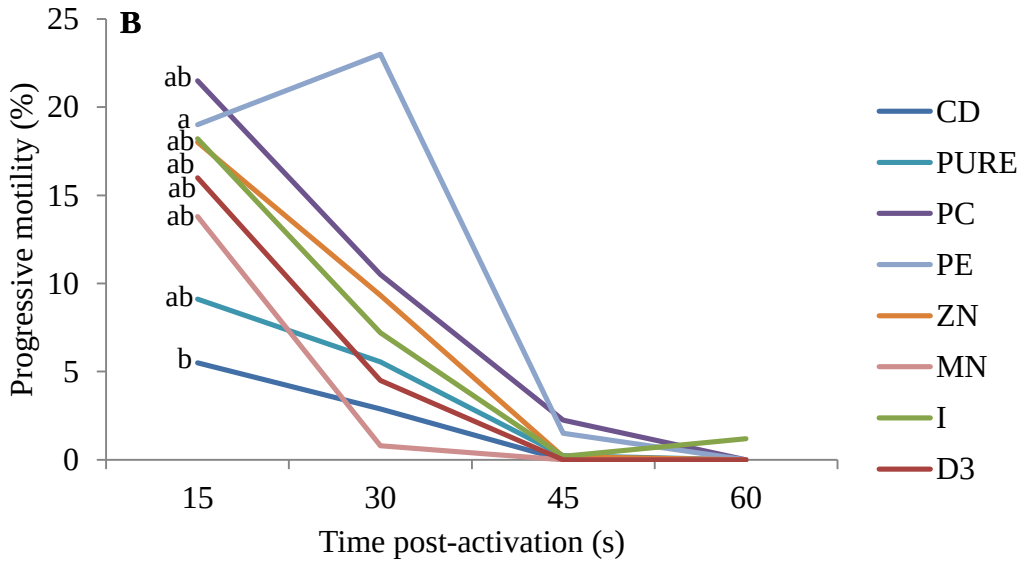
Fig. 6 – Effect of control diet (CD), purified diet (PURE) and supplemented diets (PC, PE, SE, ZN, MN, I and D3) in growth performance indexes. A – weight gain (IBW), B – daily growth index (DGI), C – average body weight (ABW), D – condition factor (CF) and E – specific growth rate (SGR). Statistical differences ($p < 0.05$) are represented with letters.

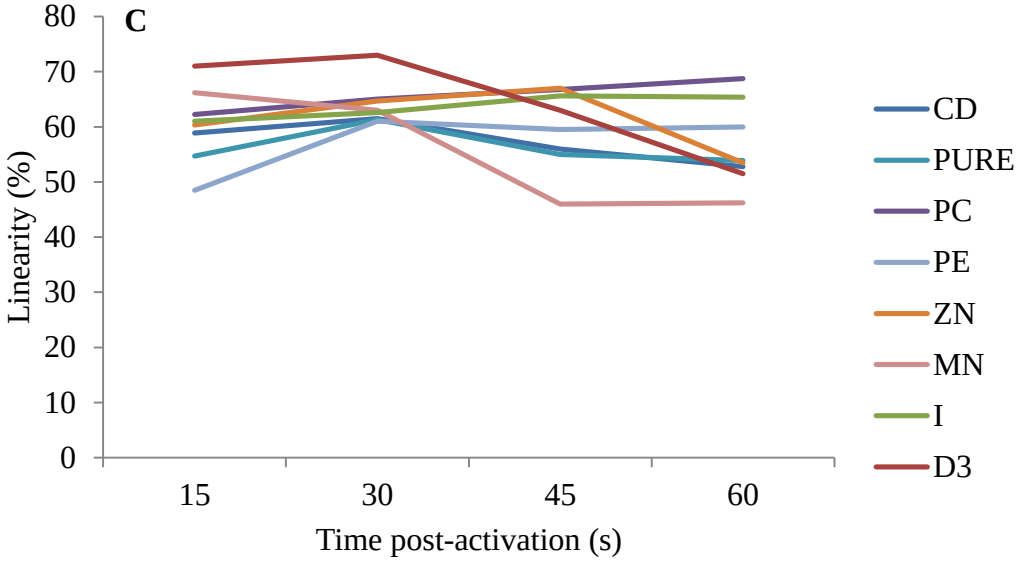
4.3 Sperm quality analysis

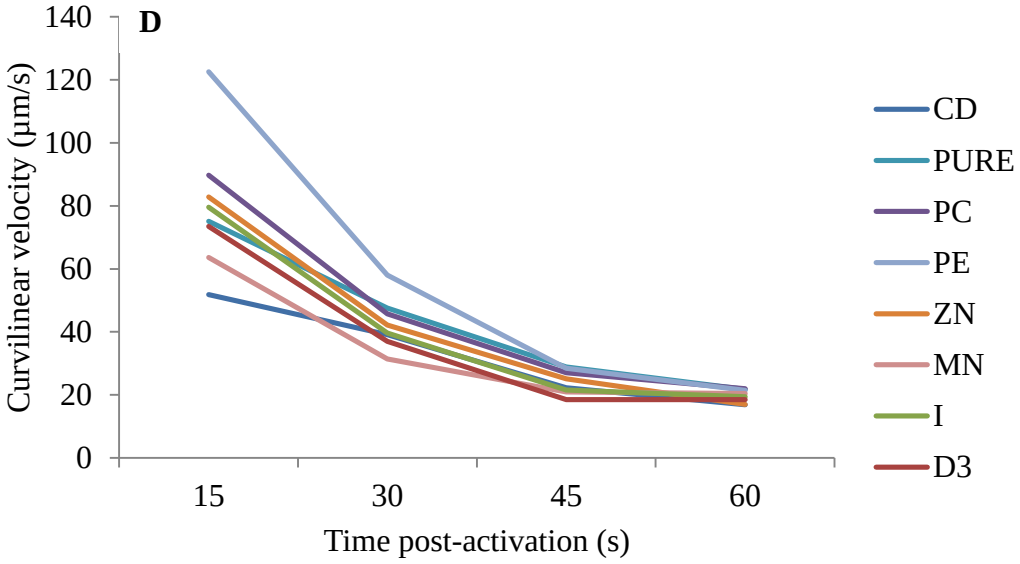
Regarding sperm analysis, several conditions of sperm were analysed, total motility (TM), Linearity (LIN), progressive motility (PM), curvilinear velocity (VCL) and straight line velocity (VSL) (Fig. 7). Differences in sperm motility were found in TM and PM, while no differences were determined in other parameters. The TM of PE diet

had significant differences (p = 0.005) regarding the CD and D3 diets, that presented the lower motility values (Fig. 7a). In terms of PM, the PE diet group presented an identical result with a better performance (p = 0.009) than the groups fed with the other dietary treatments (Fig. 7b). In the other sperm quality parameters (linearity, curvilinear velocity and straight line velocity) no significant differences (p < 0.05) were found among controls and dietary treatment diets (Fig 7 c, d, e).






C
80
70
60
50
40
30
20
10
0
Linearity (%)
15
30
45
60
Time post-activation (s)
CD
PURE
PC
PE
ZN
MN
I
D3


D
140
120
100
80
60
40
20
0
Curvilinear velocity (µm/s)
15
30
45
60
Time post-activation (s)
CD
PURE
PC
PE
ZN
MN
I
D3

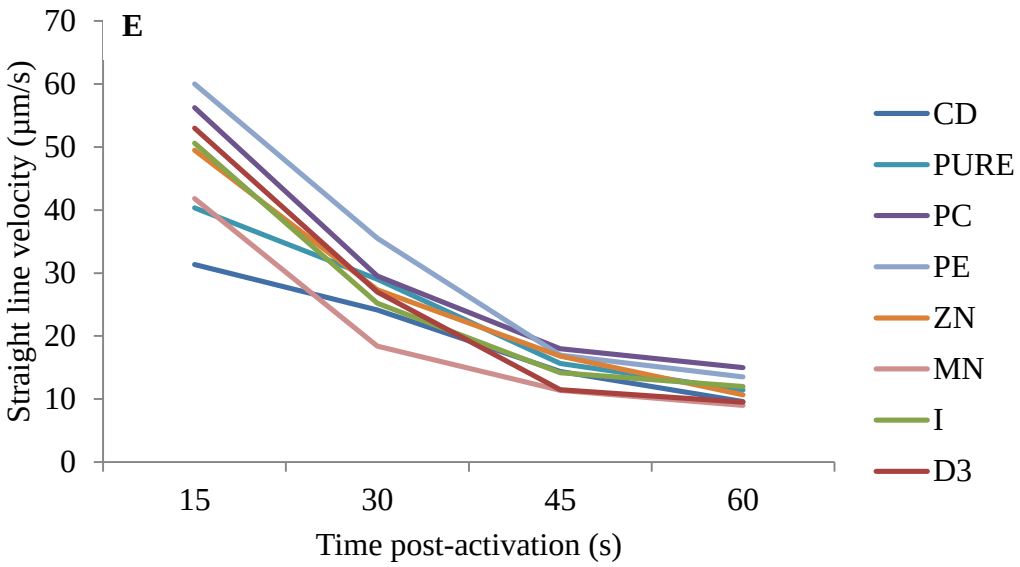


Fig. 7 – Effect of control diet (CD), purified diet (PURE) and supplemented diets (PC, PE, SE, ZN, MN, I, D3) in sperm motility of zebrafish (*Danio rerio*) at the end of the experimental period (68 days). Sperm motility parameters represented by letters, A - total motility, B - progressive motility, C – linearity, D – curvilinear velocity and E – straight line velocity. Statistical differences ($p < 0.05$) are represented with letters.

4.4 Eggs hatching rate and length

The egg quality were accessed by analysis of hatching rate, egg diameter and perivitelline space. Relatively to the egg hatching rate (Table 6), no differences were found among the control (PURE) and the remaining diets (Fig. 8). Mean hatching rates ranged between 81% and 74%, with an average among diets of 78%, excluding D3 diet that gave the lower values (38 %). The highest hatching rate were obtained by I diet with an average of 88%, but those results were not different ($p = 0.205$) from zebrafish fed with the other diets. During all the experimental procedure the CD diet did not reproduce successfully, which made impossible the analysis of eggs and larvae performance for this diet.

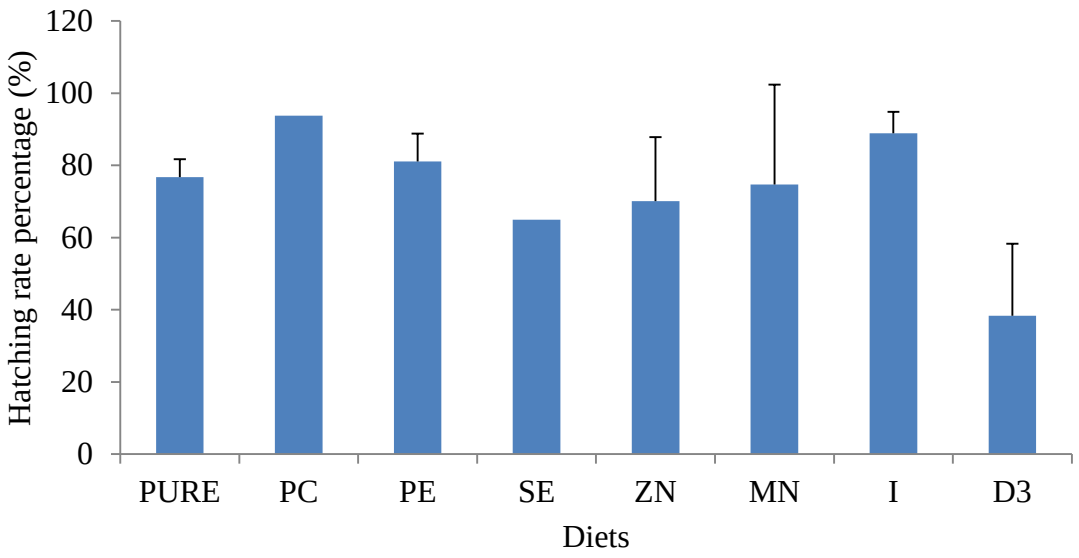


Fig. 8 - Effect of control diet (CD), purified diet (PURE) and supplemented diets (PC, PE, SE, ZN, MN, I, D3) in egg hatching rates (%) of zebrafish (*Danio rerio*). Statistical differences ($p < 0.05$) are represented by letters.

Table 6 – Dietary effects of control diet (CD), purified diet (PURE) and supplemented diets (PC, PE, SE, ZN, MN, I, D3) in the hatching rate (%) of zebrafish (*Danio rerio*) larvae. Statistical differences ($p < 0.05$) are represented with letters.

Treatment	Hatching rate
PURE	71,7
	81,7
PC	93,8
PE	73,5
	91,7
	78,0
SE	64,9
ZN	74,6
	46,3
	89,2
MN	92,2
	35,5
	96,2
I	91,9
	96,2
	80,2
	87,1
D3	58,3
	18,3

Significant differences (p = 0.0001) were observed in the egg diameter, where a significantly higher value was observed in the PE diet (1, 21 mm) comparatively with I diet (1, 13 mm) (Fig. 9a). No differences were observed between control and the others diets (Fig. 9a).

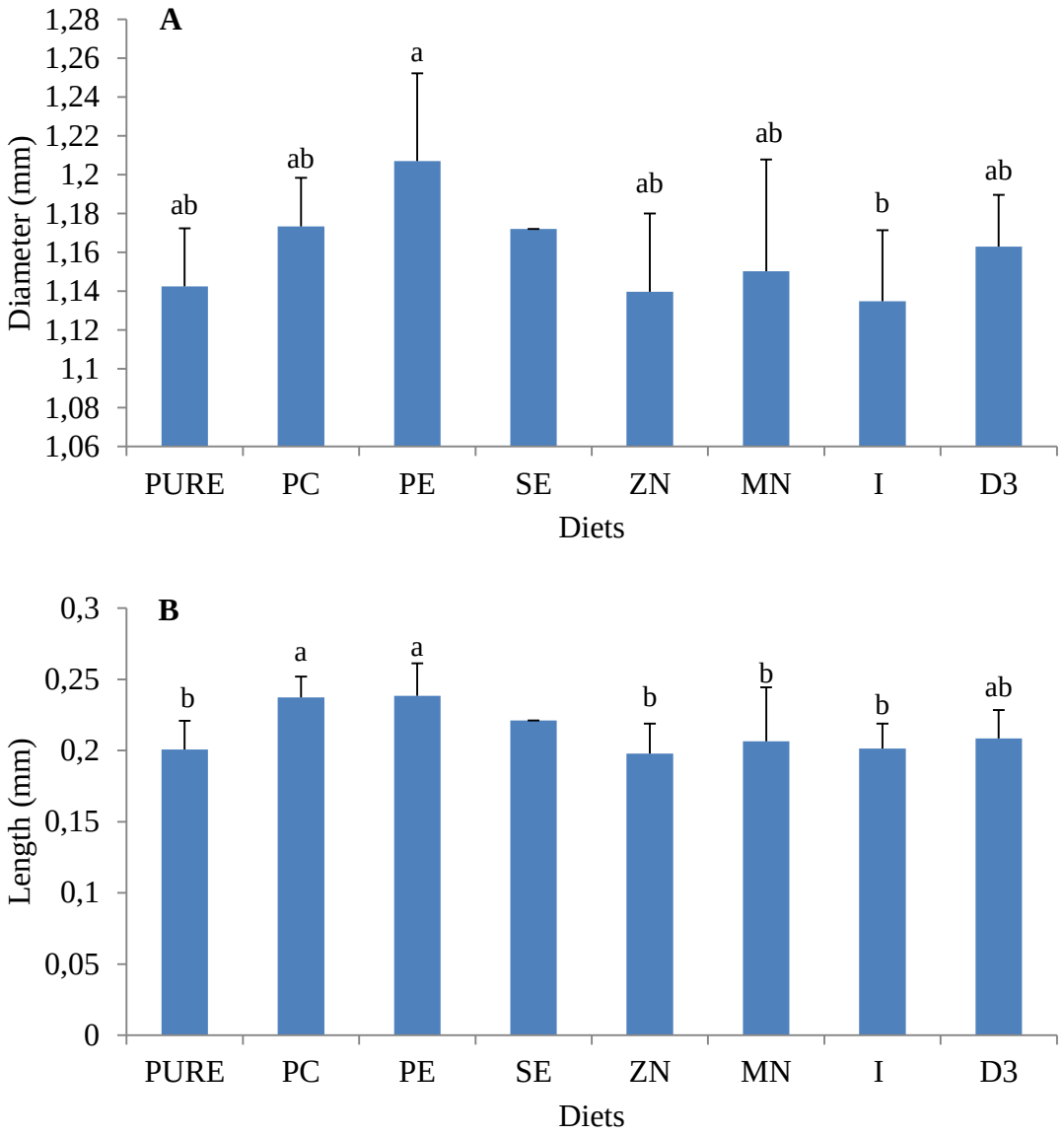
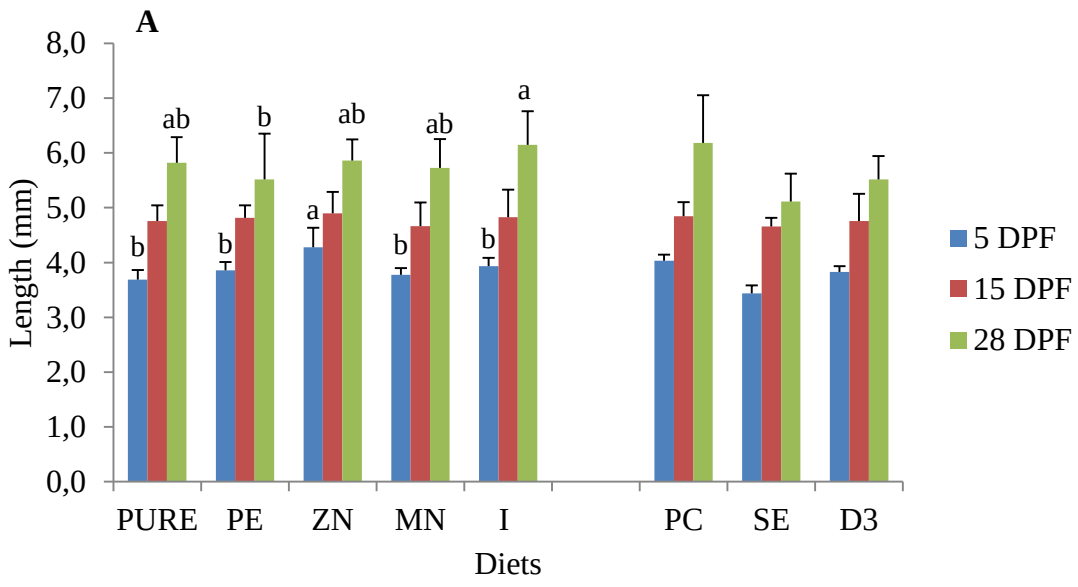


Fig. 9 – Effect of control diet (CD), purified diet (PURE) and supplemented diets (PC, PE, SE, ZN, MN, I, D3) in zebrafish egg size. The size parameters analysed are presented in A – egg diameter and B - perivitelline space. Statistical differences ($p < 0.05$) are represented with letters.

The perivitelline space also presented differences (p = 0.0001) among diets (Fig. 9b), where the PE and PC diets presented higher values (0, 24 mm) in comparison with zebrafish with the other dietary treatments, except from D3 diet. The diets MN, ZN and I presented values not significantly different from PURE diet (Fig 9b).

4.5 Larval growth in length and weight

The larvae length evaluation was done by measuring TL and SL at three points 5 days post hatching (dpf), 15 dpf and 28 dpf (Fig. 10). Regarding SL at 5dpf, the ZN diet had a significant difference (p = 0.001) to the other dietary treatments (Fig.10a). No differences were observed among larvae fed with other treatments at 5 dpf (Fig 10a). At 15 dpf no differences were found between all the diets (Fig. 10a). At 28 dpf the I diet SL showed a significant difference (P = 0.013) in comparison with the others diets (Fig. 10a). Regarding the TL identical results were found at 5dpf, where the ZN diet presented a significant different (p = 0.0001) in comparison with other diets (Fig. 10b), but no significant differences were observed at 15 dpf and 28 dpf (Fig. 10b). Larvae sampled for size were used for determining dry weight but no differences were observed at 5dpf (p = 0.696), 15 dpf (p = 0.342) and 28 dpf (p = 0.369) (Fig. 11). No differences (p = 0.397) were observed in terms of larvae survival between all the diets (Table 7).



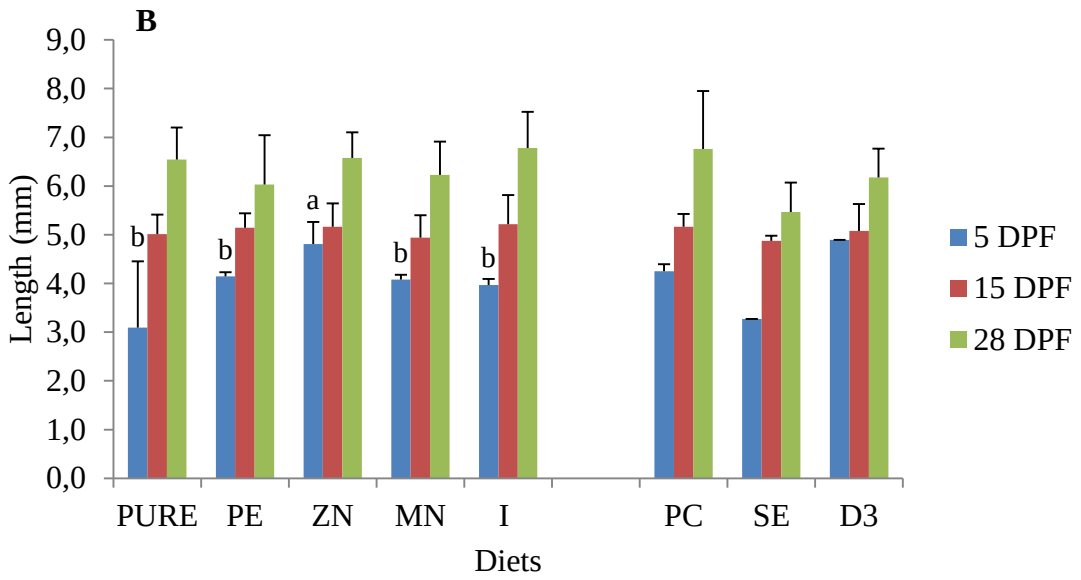


Fig. 10 – Effect of control diet (CD), purified diet (PURE) and supplemented diets (PC, PE, SE, ZN, MN, I, D3) in zebrafish (*Danio rerio*) larvae length. Samples collected in 3 periods, 5 days post fertilization (dpf), 15 dpf, 28 dpf. Larvae length (mm) parameters analysed were represented by letters, where, A - standard length (SL) and B - total length (TL). Statistical differences ($p < 0.05$) are represented with letters.

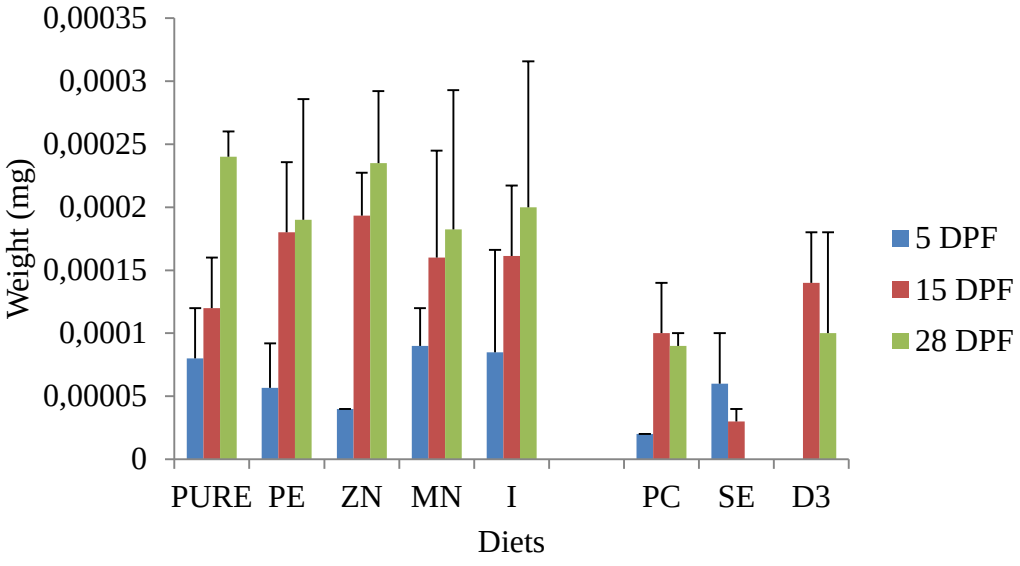


Fig. 11 – Effect of control diet (CD), purified diet (PURE) and supplemented diets (PC, PE, SE, ZN, MN, I, D3) in zebrafish (*Danio rerio*) larvae weight (mg). Samples collected in 3 periods, 5 days post fertilization (dpf), 15 dpf, 28 dpf. Statistical differences ($p < 0.05$) are represented with letters.

Table 7 – Values of survival (%), standard length (SL, mm), total length (TL, mm) and individual dry weight (DW, mg) of zebrafish (*Danio rerio*) larvae at the end of the experimental period (28 dpf). Statistical differences ($p < 0.05$) are represented with letters.

Diets	Survival	SL	TL	Individual DW
PURE	62 ± 14	5.8 ± 0.5^{ab}	6.5 ± 0.7	0.0002 ± 0.000
PC	48 ± 0	6.2 ± 0.9	6.8 ± 1.2	0.00009 ± 0.00001
PE	62 ± 17	5.5 ± 0.8^{b}	6.0 ± 1	0.0002 ± 0.0001
SE	55 ± 0	5.1 ± 0.5	5.5 ± 0.6	-
ZN	30 ± 31	5.9 ± 0.4^{ab}	6.6 ± 0.5	0.0002 ± 0.0001
MN	60 ± 31	5.7 ± 0.5^{ab}	6.2 ± 0.7	0.0002 ± 0.0001
I	62 ± 25	6.1 ± 0.6^{a}	6.8 ± 0.7	0.0002 ± 0.0001
D3	24 ± 24	5.5 ± 0.4	6.2 ± 0.6	0.0001 ± 0.0000

4.6 Mineralization analysis

The mineral deposition of phosphate was observed in larvae during the experimental procedure, with significant differences ($p \leq 0.05$) among diets (Fig. 12). The ZN diet showed the higher values (21, 99 mg/dl) in comparison with zebrafish larvae fed with other diets, followed by the PURE diet that also showed significantly higher values (16, 51 mg/dl) in comparison with diets MN (13, 63 mg/dl) and I (11, 02 mg/dl) (Fig. 12). The lower values were found in larvae fed with I diet. No differences were observed between larvae fed D3 and the other dietary treatments.

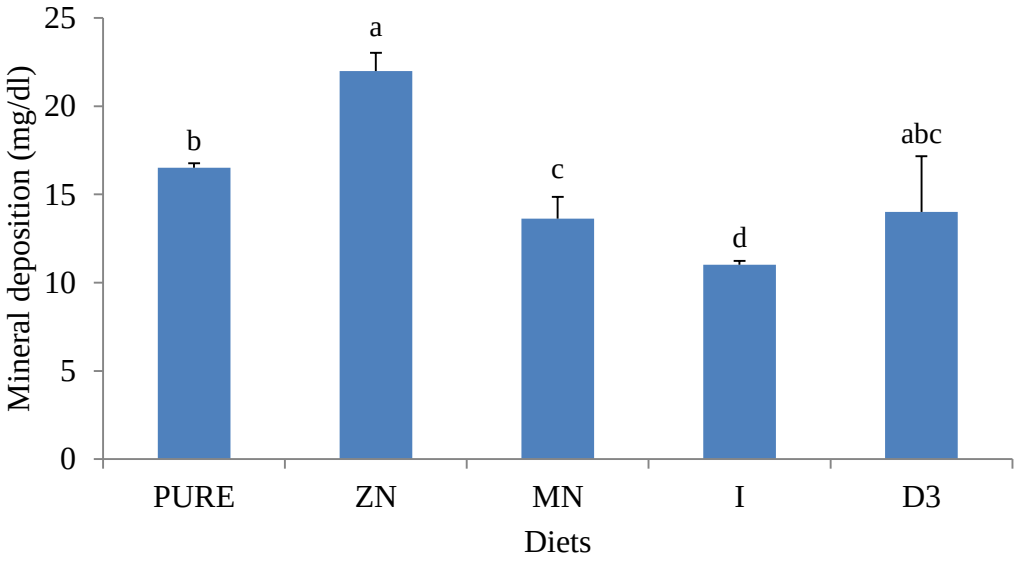


Fig. 12 – Effect of control diet (CD), purified diet (PURE) and supplemented diets (PC, PE, SE, ZN, MN, I, D3) on phosphate mineral deposition (mg/dl) in larvae of zebrafish (*Danio rerio*) at 28 days. Statistical differences ($p < 0.05$) are represented with letters.

4.7 Skeletal deformities

The evaluation of skeletal development was performed based in the osteological and meristic characteristics described by Bird and Mabee (2003). Regarding the incidence of deformities, significant differences ($p = 0.017$) were observed between the PC diet and the the remaining diets (Fig. 13). No differences were observed between the other diets used.

The charge of deformities presented by zebrafish was divided in four classes (1, 2, 3 and 4 or more deformities per fish). In the charge of deformities, no significant differences were found among diets ($p = 0.999$) (Fig. 14) although the Zn and I diets showed the lowest incidence of multiple deformities. A high number of deformed fish and of specimens with multiple deformities was observed in the SE and PC groups.

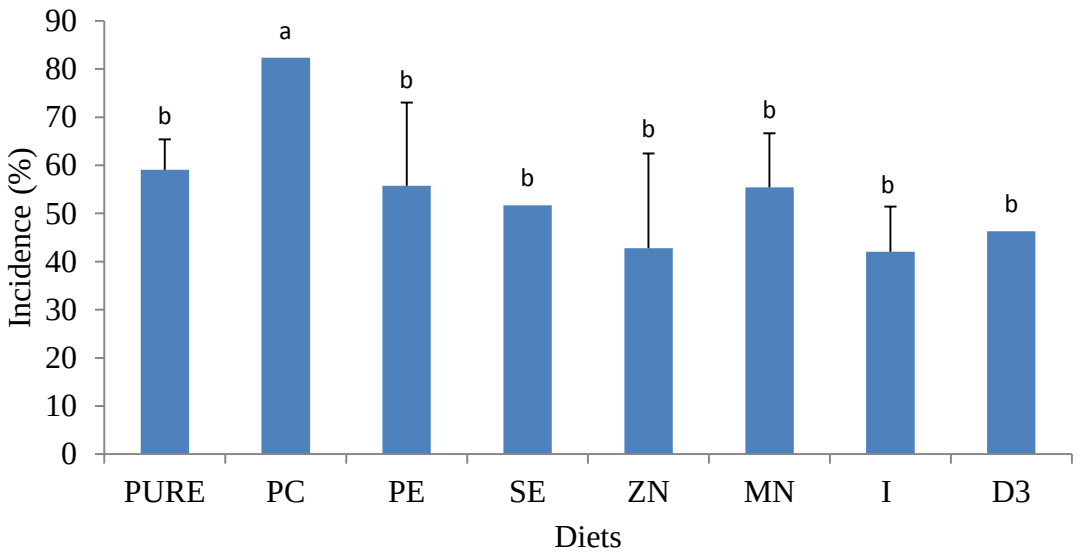


Fig. 13 – Effect of control diet (CD), purified diet (PURE) and supplemented diets (PC, PE, SE, ZN, MN, I and D3) in incidence of deformities in larval of zebrafish (*Danio rerio*) at the end of the experimental period (28 dpf). Incidence of deformities represented by DEF. Statistical differences ($p < 0.05$) are represented with letters.

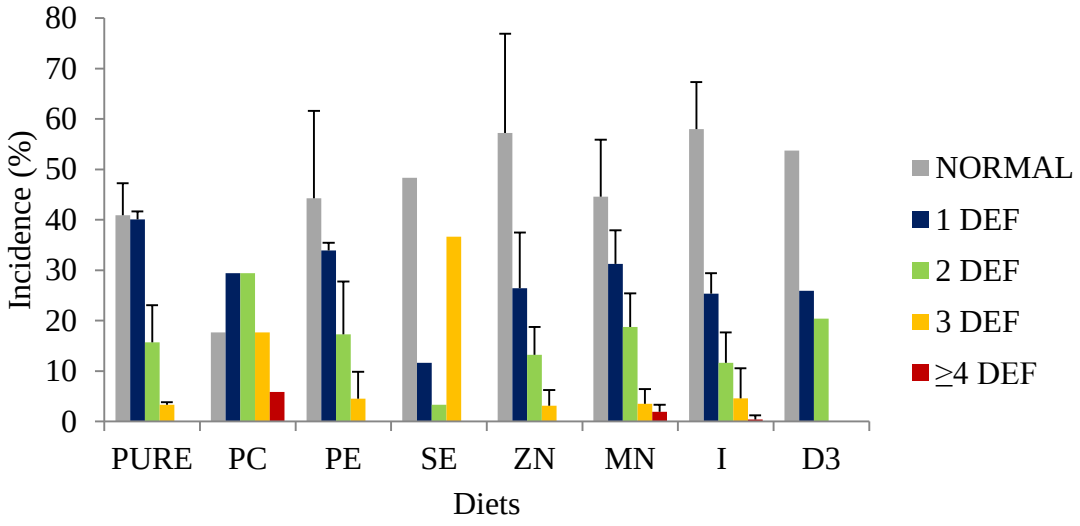


Fig. 14 – Effect of control diet (CD), purified diet (PURE) and supplemented diets (PC, PE, SE, ZN, MN, I , D3) in charge of deformities (%) in larvae of zebrafish at the end of the experimental period (28 dpf). Charge of deformities represented by 1 DEF – 1 deformity, 2 DEF - 2 deformities, 3 DEF - 3 deformities and ≥ 4 DEF - above 4 deformities. Statistical differences ($p < 0.05$) are represented by letters.

A more detailed analysis was done in order to see the differences in incidence of deformities in the different regions of the zebrafish skeleton (abdominal, caudal and caudal fin vertebrae), but without significant differences (p = 0.891) in deformities among diets, having the caudal zone, constituted by parhypural (Parhyp.) and hypurals (Hyp.) 1, 2, 3,4 and 5, the higher number of deformities (30%) (Fig. 15). The Zn and I diets presented lower values in terms of deformities, but without any significant difference (P = 0.891) in comparison with other diets. The PE and PURE diets had the higher incidences in caudal fin structures (CF Parhyp./Hyp. 1-2 and CF Hyp. 3-5), but no significant differences were found. The diets PC and SE were not considered in statistics due to the number of replicates (Fig. 15).

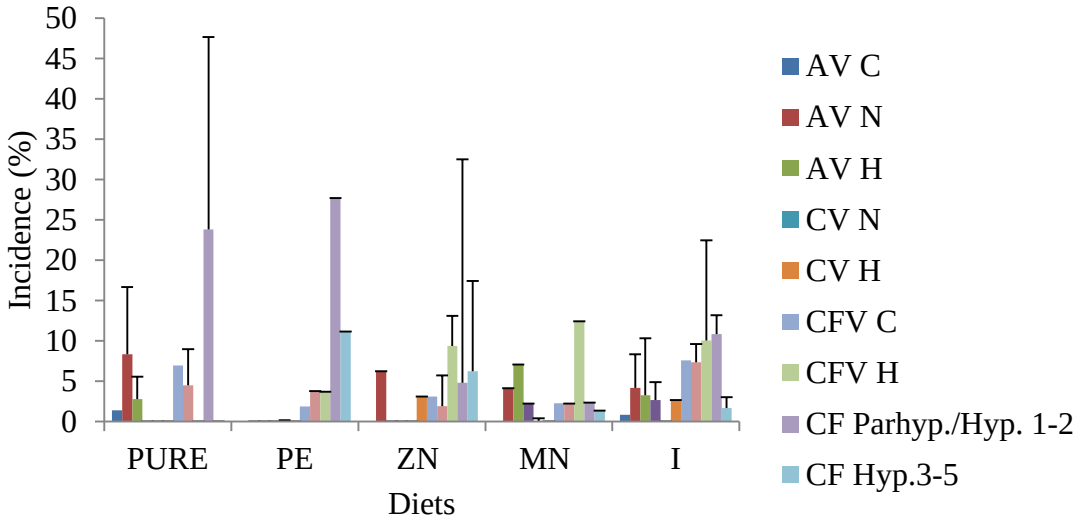


Fig. 15 – Effect of control diet (CD), purified diet (PURE) and supplemented diets (PC, PE, SE, ZN, MN, I, D3) in deformities in different zebrafish (*Danio rerio*) skeletal zones (%) at the end of the experimental period (28 dpf). Different axial skeleton of zebrafish represented by AV C - abdominal vertebra (centra), AV N – abdominal vertebra (neural), AV H - abdominal vertebra (haemal), CV C – caudal vertebra (centra), CV N – caudal vertebra (neural), CV H – caudal vertebra (haemal), CFV C - caudal fin vertebra (centra), CFV N - caudal fin vertebra (neural), CFV H - caudal fin vertebra (haemal), CF Parhyp./Hyp. 1-2 – caudal fin parhypural and hypural 1 and 2 and CF Hyp.3-5 – caudal fin hypural 3, 4 and 5. Statistical differences ($p < 0.05$) are represented by letters.

DISCUSSION

5. Discussion

5.1 Effects on growth

Zebrafish has been used as model species in several fields of biological research, including nutrition (Rubio-Aliaga, 2012). However, nutrient requirements for this species have still not been addressed, being essential to determine the importance of nutrient replenishment for reproduction, since zebrafish are continuous spawners (Markovich et al., 2007; Gonzales and Law, 2013). The bibliographic information available is contradictory relatively to protein content with authors proposing values of 46 - 50% (Ulloa et al., 2011) while others proposed a content between 31 - 60% (Siccardi III et al., 2009). Regarding carbohydrates it was proposed a content < 35% (Robison et al., 2008), and a lipid content between 5 - 34 % (Siccardi III et al., 2009). Also, the feeding methodologies used for rearing zebrafish larvae, juveniles and adults can be very diverse, being completely different among zebrafish facilities (Wilson, 2012). The zebrafish biological performances are influenced by feeding methodology, which has effects in growth, with feeding methodologies being a cause of growth dispersal. An accurate diet is required to feed larvae, being the purified diet the best option for nutritional studies in order to evaluate the effect of components and different diet formulations (Watts et al., 2012).

The results obtained in this experiment showed that adult zebrafish fed with purified diet (PURE) and purified diet supplemented with PC, and SE presented similar growth performance results to those obtained with the commercial diet (CD), with comparable lengths (Fig. 4 a, b) and weight (Fig 5). The results presented using a diet with a protein content of 60,1 % and a lipid content of 12 % are similar to those obtained by Gonzales and Law (2013), who suggested that a diet with a protein content of 59,3 % and a lipid content of 17,1 % may be the more suitable for rearing zebrafish. Despite the higher values obtained in length and weight by adult zebrafish fed with CD, but not different from PURE, PC and SE diets, those fish did not present any reproductive success. The CD diet may lack essential nutrients that are essential for reproduction, which were not observed in zebrafish fed with PURE and purified supplemented diets. The diet composition can have effects on spawning and quality of eggs produced as shown in the case of fatty acids and vitamins that are crucial factors in broodstock diets for promoting

fecundity and fertility (Markovich et al., 2007; Watts et al., 2012; Wilson, 2012). Such effect can be caused by a use of energy directed to growth instead of gonadal maturation or can be related to inter-sexual selection or even associated to size-dependent mate choice preferences, (Uusi-Heikkilä et al., 2012).

The purified diet supplemented with PC and the CD group presented an identical growth in length (Fig. 4b). This increase in growth can be associated to the feeding attractant and palatability effect, leading to an increase in the rate of fed intake (Lindsay and Vogt, 2004; Watts et al., 2012) and a consequent increase in length. Koven et al., (1998) showed that PC acts as a feeding attractant causing higher ingestion rates at a supplementation of 10 g Kg^{-1} in diets for gilthead sea bream. Seiliez et al. (2006) observed promotion of growth by increasing energy flux from the intestinal mucosa to blood in gilthead sea bream.

The diet supplemented with vitamin D3 (D3) showed the lower growth values both in length (Fig. 4 a, b) and in weight (Fig. 5). These results may be associated with a possible hypervitaminosis D, leading to negative effects in zebrafish growth, since the values used (4000 UI Kg^{-1} in diet) were higher in comparison with those used by Kaushik et al. (2011) (3000 UI Kg^{-1} in diet), without any effect . In Atlantic salmon and in channel catfish no signs of hypervitaminosis were observed in fish fed with 286,8 mg Kg^{-1} of vitamin D3 (Lock et al., 2010). Other studies have shown that excess vitamin D3 can lead to an hypercalcaemic effect, depending on the exposure time, concentrations and type of metabolite used (Srivastav et al., 1993; Lock et al., 2010). Moreover, only few studies reported inhibition of growth performance related to high dietary levels of vitamin D3 intake in fish (Lock et al., 2010). The amount used in this study with zebrafish could be excessive for the species, being necessary more studies to determine an optimal level to supply in the diet that should be lower than 4000 IU.

According to the literature, the use of growth index analysis were applied to zebrafish only regarding the weight gain (IBW), specific weight gain (SGR) and gender weight ratio (GWR) (Gonzales and Law, 2013), while other indexes like average body weight (ABW), daily growth index (DGI) and condition factor (CF) have not been not reported. In the study conducted by Gonzales and Law (2013) IBW and SGR were determined in 7 month-old zebrafish and it was obtained a weight gain of 200 mg and a SGR of 0.4 mg d^{-1} within 5 weeks of treatment using a CalaMac (Bio Marine, USA) diet. In the

present study we have observed higher values of weight gain (360 mg) and SGR (1.5 mg d^{-1}) for the CD diet in 3 month-old zebrafish (Fig. 6 a, e). The worst performances in weight gain and SGR were observed in the ZN (140 mg; 0.8 mg d^{-1}) and D3 (100 mg; 0.7 mg d^{-1}) diets (Fig. 6 a, e). According to a study performed in rainbow trout with a high dietary dose of cholecalciferol (the most active form of vitamin D3), showed to cause negative effects in weight gain by comparison with lower vitamin levels (Vielma et al., 1998). The D3 diet presented a lower performance in CF, ABW and DGI in comparison with the results obtained for the CD diet (Fig. 6b, c, d). The CF has been widely used as an indicator of health in fish (Siccardi III et al., 2009). A decline in the CF is usually interpreted as a depletion of energy reserves such as stored liver glycogen or body fat (Siccardi III et al., 2009). The present results with the D3 diet may be explained by a possible case of hypervitaminosis caused by the D3 diet. The results obtained with the ZN diet may be caused by an excess in the diet for zebrafish since the level used was 750 mg/Kg. According to the literature, the supplementation with ZN between 57 – 97 mg Kg^{-1} seems to be ideal in order to improve growth performance in Atlantic salmon fry, however in the same study, a supplementation up to 1000 mg Kg^{-1} showed similar growth results (Nguyen et al., 2008). The results obtained in this study support the idea that diet composition has strong effects on growth and condition of zebrafish and also that age may influence those results.

5.2 Sperm quality analysis

The production of high quality gametes is associated to an achievement of high fertilization rates, but also involved in the development of healthy larvae (Rurangwa et al., 2004). The evaluation of sperm quality is essential in order to evaluate the success of reproduction. The sperm motility has been used as general criteria for sperm quality evaluation (Cabrita et al., 2009). Most of the teleost species present an external fertilization where gametes are release to the water (Cabrita et al., 2009), a hazardous medium that can damage sperm cell membrane (Diogo et al., 2010). Sperm cell membranes can be affected by lipid peroxidation caused by reactive oxygen species (ROS) than can compromise sperm functionality (Lahnsteiner et al., 2009), reduce fertilization ability (Bansal and Bilaspuri, 2011; Martínez-Páramo et al., 2012b) and increase susceptibility to DNA damage (Słowińska et al., 2013). Sperm cell membranes

are mainly composed by phospholipids that are essential for protection against oxidative damages induced by ROS (Martínez-Páramo et al., 2012b; Słowińska et al., 2013). Certain species like zebrafish and European sea bass, present a sperm membrane where the two most abundant phospholipids classes are PC and PE, that corresponds to 80% of total sperm membrane lipid content (Martínez-Páramo et al., 2012b).

The composition of sperm plasma membrane is known to change during reproductive season in terms of lipid concentration (Martínez-Páramo et al., 2012b), as a consequence of broodstock nutrition manipulation in lipid composition (Bobe and Labbé, 2010) that results in a change of sperm quality (Martínez-Páramo et al., 2012b). The introduction of essential fatty acids in broodstock nutrition and consequent reflexes in gametes were observed in several species such as rainbow trout (Watanabe et al., 1983; Hamre et al., 2013), European sea bass (Asturiano et al., 2001), and gilthead sea bream (Fernández-Palacios et al., 1995, 1997). In the present study we found identical results, with the supplementation of diets having an influence in fish development and sperm motility parameters. Adult zebrafish supplemented with PE presented a significantly better sperm quality revealed by a higher total (TM) and progressive (PM) motility in comparison with the other diets, but only significant relatively to the D3 diet (Fig. 7a, b). The introduction of PE in diet had a positive effect that may be related to the composition and integrity in the lipidic bilayer of plasma cell membrane, since it represents one of the major classes of phospholipids in plasma membrane, causing effects on sperm movement durability (Martínez-Páramo et al., 2012b).

Adult zebrafish fed with D3 diet presented the lowest values for TM (Fig. 7a), possibly associated to a hypervitaminosis D, as discussed. The CD diet had the lower PM values (Fig. 7b) in comparison with other diets that can help explain the failure to reproduce. The CD diet was supposed to provide a ω6/ ω3 fatty acid ratio that would be favourable for the fertilization rates and spawning performance of adult zebrafish as described by Gonzales and Law (2013), but since it was not formulated specifically for zebrafish it may be nutritionally imbalanced for the species.

In the present study, PE supplementation showed positive results in TM and PM (Fig. 7a, b), which supports the importance of this phospholipid in zebrafish sperm motility, where highly motile sperm are associated to a better chance of fertilization in

comparison with spermatozoa presenting lower movement capacity (Rurangwa et al., 2004).

5.3 Eggs hatching rate and size

High quality eggs are essential for high fertilization rates in species such as gilthead sea bream and are directly dependent of maternal nutritional delivery, with the fulfilment of nutritional requirements associated to good quality eggs (Gonzales and Law, 2013), spawn quantity and frequency (Bobe and Labbé, 2010). The egg nutritional quality is also an essential factor in hatching rates and consequent larval development (Migaud et al., 2013). The egg hatching rate, embryo diameter and perivitelline space have been previously used to evaluate egg quality. In many species there is a relationship between female body size and fecundity (Uusi-Heikkilä et al., 2010). Small size females might have a compensation process due to the reduced fecundity and spawning frequency producing eggs with larger diameter compared with larger females (Uusi-Heikkilä et al., 2010), although, the exact mechanism that leads to this remains unclear. Despite larger egg size small females tend to produce eggs with larger perivitelline space or thicker chorion rather than larger yolk material (Uusi-Heikkilä et al., 2010). The present data does not show a correlation between the small adult zebrafish and the size of the eggs, as obtained by Uusi-Heikkilä et al. (2010), since the results obtained with PE presented the higher egg size values (Fig. 9a).

In our results, eggs from PC and PE had the the largest values of perivitelline space (Fig. 9b). An explanation to this fact can be the importance of phospholipids in fish development, as energy source and for promoting a correct embryonic and larval development (Tocher, 1995; Tocher et al., 2008). The importance of PE in egg composition has been described in a range of species, Atlantic herring (*Clupea harengus*), cod (*Gadus morhua*), halibut (*Hippoglossus hippoglossus*) and plaice (*Pleuronectes platessa*) (Tocher, 1995; Tocher et al., 2008).

The smaller egg diameter and perivitelline space were obtained for the I diet (Fig 9a, b). Interestingly, we have observed the higher larval growth with iodine supplementation. A study performed with enrichment of artemia with iodine showed minor or small effects in Atlantic halibut larval growth performance (Hamre et al., 2013).

There is a correlation between small fish producing larger and lower quality eggs with an increased egg mortality rate in comparison with eggs produced by large females (Uusi-Heikkilä et al., 2010). However, this correlation was not observed since no differences were observed between small and large females due to the mortality obtained during all the experiment. Other studies support that the size of the egg is not always linked to its quality and that eggs with different sizes can exhibit a similar development as observed in species such as rainbow trout and European sea bass (Migaud et al., 2013). Despite that, the size of the eggs can be related to the future development of the embryo (Bobe and Labbé, 2010). According to Markovich et al. (2007) the dietary intake by adult zebrafish can significantly impact the number of eggs produced, the hatching rate and the larval length. In the present study no differences were obtained in the number of eggs laid between larger females and smaller females.

5.4 Larvae growth

The larval growth and development are conditioned by the different rearing conditions (Wilson, 2012). Changes in temperature are one of the principal factors causative of variations in fish growth, as studied in several species (Georgakopoulou et al., 2007, 2010). However there is an effect of different live feeds, like commercial diets (CD) or supplemented diets that can be observed by the consequent variations of fish growth (Gómez-Requeni et al., 2010; Ulloa et al., 2011; Wilson, 2012).

Zinc supplementation is associated to bone formation and mineralization. A study performed by Nguyen et al. (2008) showed that supplementation of ZN promotes normal skeletal development. Other studies reported the improvement of growth performance in Atlantic salmon with 57-97 mg Kg^{-1} of ZN supplementation (Nguyen et al., 2008). In the results obtained in this study, for a dosage of 750 mg Kg^{-1} it was observed an increment in larvae standard and total length until the onset of exogenous feeding at 5dpf in comparison with other diets (Fig. 10a, b). However, no differences were observed between ZN and the other diets at the middle and end of the experiment (15 and 28 days). These results may be representative of the importance of ZN for the development of larvae in the first stages of life, while in later life stages there must be a lower Zn requirement.

Freshwater fishes present a lower availability of I in comparison with saltwater species (Hawkyard et al., 2011). It is known the importance of I for the production of thyroid hormones (THs), that are strongly associated to larvae development and bone ossification as in the case of zebrafish (Hawkyard et al., 2011). Studies conducted in zebrafish showed a relationship between the inhibition of THs during larvae development causing problems as reduced fin development, scale formation and affecting pigmentation (Hawkyard et al., 2011). In this study it was observed that I supplementation at 20 mg Kg^{-1} affected standard length, which had the higher values at 28 dpf, in comparison with the diet containing phospholipid PE (Fig. 10a). A study performed in cod larvae with enrichment of rotifers with I, showed that levels close to 129 mg Kg^{-1} DW are toxic to larvae (Penglase et al., 2013). Iodine supplementation close to 26 mg Kg^{-1} was found to be ideal for cod larvae (Hamre et al., 2013; Penglase et al., 2013), a value similar to the used here for zebrafish, with good growth performance, indicating that the values used must be close to the ideal for promoting zebrafish larval growth.

The lower values in obtained by PE supplementation on diet in standard length (SL) at 28 dpf in comparison with the values of the I diet are in controversy with the results obtained in marine species like European sea bass and turbot, were it was showed that different dietary phospholipids classes affected positively the growth and larval development (Geurden et al., 1997, 1998). Those differences could be associated to the different environments or even the rearing conditions.

Several studies were conducted where larvae length is used to evaluate different diets. Goolish et al. (1999) reported a survival of 60 % and a standard length (SL) of 6.4 mm at 21dpf using processed diets as larval food. Other studies revealed that paramecia may not be suitable for rearing zebrafish, since low length values of 4.7 mm at 28 dpf were achieved with this feeding procedure in comparison with fish fed with artemia and commercial diets. Better results were obtained by Carvalho et al. (2006) by feeding zebrafish with artemia that reached 14.3 mm at 21 dpf with an 86 % survival. However a lower length (6.9 mm) and survival (55 %) was achieved using a purified diet.

In the present study we achieved results similar to the ones of (Goolish et al., 1999) with an average of 6.4 mm of total length (TL) at 28 dpf and a survival of 61 %, for zebrafish larvae feed with an experimental purified diet (PURE) (Table 6).

Nevertheless, the results were not different from those obtained by Carvalho et al. (2006) using purified diets. The diets supplemented with PE and I presented 62 % of survival (Table 6). The different results obtained for zebrafish larvae are indicative of discrepancies in feeding and rearing conditions and in the densities used, where zebrafish larvae can be directly affected by nutritional conditions and feeding frequency (Gómez-Requeni et al., 2010; Ulloa et al., 2011; Wilson, 2012).

5.5 Larvae mineralization analysis

The zebrafish has been intensively studied as a model for vertebrate development, with an important focus on bone and scale formation (Witten et al., 2001). Bone resorption of zebrafish occurs essentially to cover the demands of growth and any manipulation affecting growth will have effects on the performance of bone resorption (Witten et al., 2001). The importance of ZN in fish larvae development is well known, with negative effects such as structural deformities caused by ZN deficiencies (Nguyen et al., 2008). Studies performed in rat showed that ZN supplementation in diet increased bone strength (Ovesen et al., 2001) and Zn supplementation in diet has been associated to a stimulatory effect on bone formation and mineralization in fish. Zn was shown to have a positive effect on Atlantic salmon larvae growth fed with 57-97 mg Zn Kg^{-1} in diet (Yamaguchi, 1998; Nguyen et al., 2008). However, a supplementation with 1000 mg Kg^{-1} also presented similar results (Nguyen et al., 2008). In the present study an higher mineral deposition were obtained with the Zn diet in comparison with PURE, MN and I diets, but not different from D3 (Fig. 12).

Vitamin D3 (D3) is known to have an important role in regulation of calcium and phosphate homeostasis (Anderson and Atkins, 2008). The importance of D3 on bone mineralization was reported in fish by Fenwick et al. (1994) in experiments performed in emerald rockcod (*Pagothenia bernacchii*) by ip injection, with an increase of mineralization by 150%. Other studies were performed with addition of vitamin D3 to rearing medium, with an reported increase in bone mineralization (Fleming et al., 2005). However, bone catabolic effects were also reported in fish (Lock et al., 2010). In the presented results the D3 diet had similar mineral deposition to the ZN diet, but showed

lower values in terms of adult zebrafish growth and reproduction, as described before (Fig.12).

The larvae fed the I diet had the lower values of mineral deposition (Fig. 12). Those results could be related to the indirect role presented by iodine, since it functions as a regulator of fish metabolic activity and a precursor of THs (Watanabe et al., 1997) production, therefore affecting bone mineralization. However, despite the lower levels of mineralization, iodine presented higher growth in length at 28 dpf, which may indicate an indirect role of I in zebrafish growth.

5.5 Larvae deformities

The presence of deformities in the first stages of development is very common in reared species and can be related to rearing conditions (Boglione et al., 2013b; Gavaia et al., 2009; Koumoundouros et al., 1997). During the skeletal development several regions of the vertebral column can be affected by the rearing conditions that fish are submitted. In addition deformities can be related to diet composition, with effects on growth, metabolism, disease and longevity already described for several produced species.

The zebrafish has been used as an effective model to study mineralization characteristics of skeleton and for the study of bone diseases, (Siccardi III et al., 2010). However, few studies were conducted to determine possible deformities associated to nutritional requirements in zebrafish (Siccardi III et al., 2009). The presence of deformities in zebrafish were mainly observed for characterizing transgenic lines, as the case of din- mutants that displayed anomalies as duplicated ventral fin lobe, variably reduced ventral tail fin lobes and tail fins (Fisher and Halpern, 1999). Recent results regarding wild type lines were achieved by Bensimon-Brito et al. (2010), who reported that vertebrae adjacent to the urostyle, are highly susceptible to fusion in comparison with trunk vertebrae. The effects of diet supplementation and further consequences on zebrafish skeletal development were not performed yet.

Several studies reported the important role of ZN, being associated to a proper embryonic development and essential to normal growth and skeletal formation (Yamaguchi, 1998; Ovesen et al., 2001; Yamaguchi and Fukagawa, 2005; Nguyen et

al., 2008). Supplementation with Mn had positive effects in growth and in the promotion of normal skeleton development in red sea bream larvae, fed with supplemented artemia nauplii with a concentration from 12 – 43 mg Mn Kg^{-1} (Nguyen et al., 2008).

Iodine deficiency has been suggested to be associated to larval deformities, but no studies had any results in decreasing deformities by iodine supplementation (Boglione et al., 2013b). Though, other diet supplements do not seem to be crucial in reducing deformities, as is the case of PC, where a decrease in the ratio did not show an increase in skeletal deformities (Sandel et al., 2010).

In the present study the zebrafish skeleton was evaluated in order to analyse the effect of diet supplements and their effects on skeletal development. The increment of PC (5g kg^{-1}) on diet caused an increased (80%) incidence of deformities in comparison with the other diets (Fig. 13). The presented results are similar to those obtained by Geurden et al. (1997;1998), in which stated that PC is the less effective phospholipid in reducing deformities. However, Cahu et al. (2003) observed that a 2.18 PC/PI ratio (1.6% of DW) prevented European sea bass deformities during larval development. Recently a study performed by Sandel et al.(2010), showed that decreased levels of PC did not cause an increase of deformities in gilthead sea bream larvae. In the same study, the higher values of PC (5.7 g $100g^{-1}$ DW on diet), presented the lowest percentage of deformities, but without significant differences among the other formulated diets, which presented lower values of PC. No differences were observed regarding the deformities charge in different diets (Fig. 14) or the distribution of deformities across the different structures (Fig. 15).

Despite no significant differences in the incidence of deformities between other diets, ZN and I diets presented lower values in terms of deformities. The observed results in zebrafish larvae fed with ZN diet can be associated to larvae growth, where the higher initial growth were observed at 5dpf. These higher development rate in comparison with the other diets could promote the occurrence of deformities during this phase (Boglione et al., 2013b). Interestingly, the ZN diet had the higher mineral deposition observed (Fig. 12), such result could be another explanation to the lower incidence of deformities in that treatment group. ZN have a stimulatory effect in bone formation and mineralization (Nguyen et al., 2008) that should be reflected in bone strength and in an

optimal skeletal development, as observed in mammals (Ovesen et al., 2001). Similar results were observed with the I diet, which presented similar incidence of deformities, but with the lowest mineral deposition. Interestingly, at 28 dpf the I diet presented the higher values in larvae length. The higher larvae growth could be one of the factors that condition development and can affect the incidence of deformities (Boglione et al., 2013b). Iodine is a regulator fish metabolic activity and a precursor of THs, that influences fish growth and bone mineralization (Watanabe et al., 1997) and that can be positively influencing the morphogenesis.

CONCLUSIONS

6. Conclusions

The common practices in different zebrafish facilities, of feeding zebrafish with commercial diets and live feed without a balanced and controlled diet are susceptible to interfere in scientific results compromising the results and replication. Several nutritional studies are required to establish an adequate diet for zebrafish, since this is a species with high importance for research.

According to the presented results PURE diet having a lower lipid content had identical results to other diets used with higher lipid content and seemed to be suitable for rearing zebrafish, which make this diet very close to the ideal values needed by zebrafish. The several supplements caused different effects in adult and larval zebrafish.

The phospholipid PC had good results in terms of adult length and an increase egg diameter and perivitelline space, but had consequences in larvae deformities. The PE had a good impact in sperm total motility (TM) and progressive motility (PM) as higher values for perivitelline space, but with the worst results in larvae growth in length. Those results support that a diet with similar concentration must be used to increase sperm motility and consequent fertility rates.

The zinc (ZN) had positive results in initial larvae growth and mineral content, which seems that ZN has a crucial importance in the larvae development during the first 5 days post fertilization (dpf) where larvae are dependent of yolk reserves. Iodine (I) had good results in terms of larvae length at 28 dpf, but with lower values in egg diameter, pervitelline space and mineral content, indicating that the I diet had an effect in larvae growth between 15 – 28 dpf, where differences were higher in comparison with other diets. Similar values should be used in larval diet in order to promote growth in length.

The Vitamin D3 (D3) had negative effects in adult zebrafish growth and sperm total motility (TM). The supplementation of 4000 IU of D3 on diet is higher in comparison with other studies which support a possible hypervitaminosis, leading to negative consequences in fish development and fish reproduction.

In conclusion, it was observed that supplementation in mineral like ZN that had a positive effect in larval skeletal development and mineral deposition but not in adult zebrafish growth performance or reproduction. This highlights the importance of

different diet formulations in specific periods of the zebrafish life cycle, where supplementation of specific compounds could be a solution in order to optimize zebrafish rearing. More studies regarding zebrafish nutrition are needed in order to design balanced diets that allow a rapid growth of both adults and larval stages and without compromising scientific results, as required by the scientific community.

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