

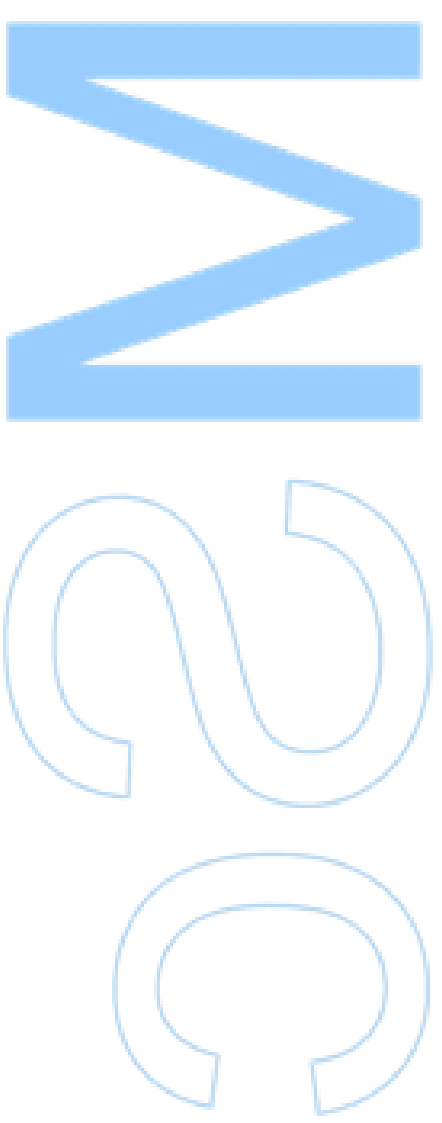
Evaluating the effect of dietary nitrate supplementation on growth, oxygen consumption and reproductive performance of zebrafish (*Danio rerio*)

Inês Gomes Campos
Masters Degree Dissertation presented to
Faculty of Sciences, Porto University, in
Aquatic Biologic Resources
2013

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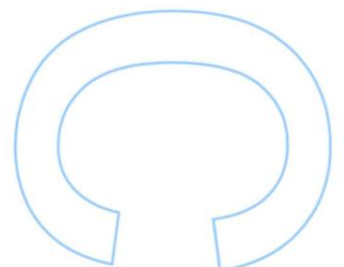
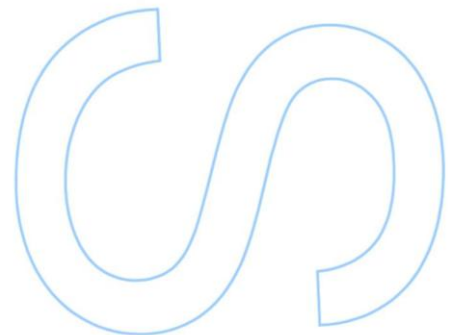
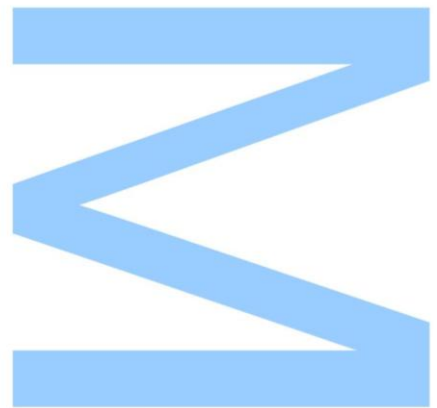




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Master of Sciences in Aquatic Biologic Resources
Department of Biology
2013

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

O Presidente do Júri,

Porto, ____/____/____

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Abstract

Nitrate and nitrite have been long considered hazardous substances for human and animal health. However, recent research has shown multiple benefits of dietary nitrate, mostly attributed to the reduction of ingested nitrate into nitric oxide, a substance known for the control of several physiologic functions. Nonetheless, even though the recently suggested beneficial effects of ingested nitrate have been widely studied in mammals, little is known about this subject on fish. The present study was conducted in order to assess the effect of dietary nitrate supplementation on zebrafish (*Danio rerio*) growth, reproductive performance and oxygen consumption. Three trials took place successively to analyze each one of those parameters by the mentioned order, using the same set of fish throughout the whole study. Four levels of sodium nitrate (1, 2, 4 and 8%) were tested as a nitrate supplementation source on the diets used in this assessment, against a control diet with no supplementation. Dietary nitrate supplementation showed no improvement in zebrafish growth, but at higher supplementation levels (4 and 8% of sodium nitrate) led to a decreased growth performance, which indicates a toxic effect of nitrate at these levels. Regarding the reproductive performance, nitrate affected spawning frequency positively at a low level (1% sodium nitrate) but showed toxic effects at a high level (8% sodium nitrate). Oxygen consumption was lower in fish fed diets with intermediate nitrate levels (2 and 4% sodium nitrate). Results corroborate previous findings that dietary nitrate promotes an oxygen sparing effect, and broaden this effect to fish, which, to our knowledge, has not been reported yet.

Keywords: nitrate; nitric oxide; growth; oxygen consumption; reproduction; zebrafish.

Resumo

O nitrato e o nitrito têm sido desde há muito tempo considerados substâncias perigosas para a saúde humana e animal. Contudo, estudos recentes têm vindo a mostrar múltiplos benefícios do consumo de nitrato, provavelmente associados à redução do nitrato ingerido a óxido nítrico, uma substância conhecida pelas suas inúmeras funções a nível fisiológico. No entanto, apesar de os benefícios da ingestão de nitratos terem sido ultimamente bastante estudados em mamíferos, pouco se sabe ainda sobre este tópico em peixes. O presente estudo foi realizado com o objetivo de determinar o efeito da suplementação dietária de nitrato no crescimento, performance reprodutiva e consumo de oxigénio do peixe-zebra (*Danio rerio*). Foram realizados três ensaios sucessivos para analisar cada um dos parâmetros, pela ordem supramencionada. Quatro níveis de nitrato de sódio (1, 2, 4 e 8%) foram testados como fonte de suplementação de nitrato nas dietas utilizadas nesta investigação, contra uma dieta de controlo sem suplementação. A suplementação das dietas com nitrato não promoveu melhorias no crescimento do peixe-zebra, mas os níveis mais elevados de suplementação (4 e 8% de nitrato de sódio) conduziram a um decréscimo da performance de crescimento, o que indica um efeito tóxico do nitrato a estes níveis. No que diz respeito à performance reprodutiva, o nitrato afetou positivamente a frequência de desova quando incorporado num nível baixo na dieta (1% de nitrato de sódio), mas revelou-se tóxico num nível elevado (8% de nitrato de sódio). O consumo de oxigénio foi menor nos peixes alimentados com níveis intermédios de nitrato (2 e 4% de nitrato de sódio). Os resultados corroboram a descoberta de que a ingestão de nitrato promove um efeito de poupança de oxigénio, e estendem esse efeito aos peixes, o que, do nosso conhecimento, nunca havia sido referido.

Palavras-chave: nitrato; óxido nítrico; crescimento; consumo de oxigénio; reprodução; peixe-zebra.

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List of abbreviations

ABW – average body weight

ADI – acceptable daily intake

ANOVA – analysis of variance

BW – body weight

CVDs – cardiovascular diseases

DGI – daily growth index

FAO – Food and Agriculture Organization

FE – feed efficiency

O₂ – oxygen

NO – nitric oxide

NOS – nitric oxide synthase

SCF – Scientific Committee on Food

WHO – World Health Organization

1. Introduction

1.1. Dietary nitrate: foe or friend?

Nitrate and nitrite have usually been considered harmful substances for human and animal health and there are strict regulations considering their concentrations in food and water for consumption. According to the World Health Organization (WHO), nitrate consumption has often been related to increased risk of gastric cancer, congenital malformations, and childhood methaemoglobinaemia and diabetes mellitus, among other illnesses in humans. However, WHO also states that none of these conditions have been positively correlated to nitrate ingestion, as the data collected from scientific studies don't show a significant causal connection. The guideline value set by this organization for nitrate in drinking water, 50 mg/L as nitrate ion, was based on evidences from epidemiologic studies on methaemoglobinaemia in bottle-fed infants. According to these epidemiological evidences, this disease was not reported in infants in areas where drinking water contained less than 50 mg of nitrate per liter. Nonetheless, evidences show that the risk of methaemoglobinaemia is enhanced in the presence of simultaneous gastrointestinal infections, even suggesting that this could actually be the primary cause of methaemoglobinaemia (World Health Organization, 2011). The Acceptable Daily Intake (ADI) of nitrate for humans, 3.7 mg/kg body weight/day, has been established since 1962 by the WHO based on a few experiments in rats and dogs (Katan, 2009) and reconfirmed by the Joint FAO/WHO Expert Committee on Food Additives in 2002 (European Food Safety Authority, 2008).

In the very recent years, however, health benefits of nitrate ingestion have been shown at concentrations until now considered toxic. These benefits are specially related to reducing the risk of cardiovascular diseases and improving athletic performance. Some studies have shown that nitrate may be the source of vegetables and fruits cardiovascular protective effects, and that a reference blood pressure-lowering diet leads to a daily intake for a 60kg adult about 550% higher than the recommended value by WHO (Hord *et al.*, 2009). Other researchers have shown that nitrate ingestion can improve cardiovascular health, whether by reducing blood pressure (Sobko *et al.*, 2010; Vanhatalo *et al.*, 2010; Carlstrom *et al.*, 2011; Hobbs *et al.*, 2012) or by preventing peripheral artery disease and tissue ischemia (Garg & Bryan, 2009; Carlstrom *et al.*, 2011; Kenjale *et al.*, 2011; Lavu *et al.*, 2011; Allen *et al.*, 2012). Nitrate ingestion was also found to improve mitochondrial efficiency (Larsen *et al.*, 2011) and muscle efficiency (Bailey *et al.*, 2010; Vanhatalo *et al.*, 2011), which

may explain the results obtained using nitrate as an improver of athletic performance, either by reducing the oxygen spent during exercise (Larsen *et al.*, 2007; Bailey *et al.*, 2009; Bescos *et al.*, 2010; Larsen *et al.*, 2010; Lansley *et al.*, 2011b) or by maximizing the power obtained from a certain amount of oxygen spent (Kenjale *et al.*, 2011; Lansley *et al.*, 2011a). Furthermore, and not so recently, nitrate ingestion was also associated with the defense against some gut pathogens (Dykhuizen *et al.*, 1996; Dykhuizen *et al.*, 1998).

In view of all these beneficial effects, recently some authors have claimed that nitrate (as well as nitrite) should be considered nutrients (Hord *et al.*, 2009; Larsen *et al.*, 2010), even suggesting that the present regulation levels for nitrate ingestion may actually be restricting our nitrate intake to a deficient dietary level (Hord, 2011) and that the propaganda against nitrate may be not protecting us from potential harmful effects but instead preventing the consumption of an essential nutrient (Lundberg, 2009; Milkowski *et al.*, 2010).

The most plausible and recent hypothesis suggests that beneficial effects of the dietary inorganic nitrate are not ascribed to nitrate itself but to nitric oxide (NO) resulting from the reduction of nitrate inside mammal's body (Hord, 2011; Larsen *et al.*, 2011), a well-known signaling molecule implicated in the regulation of many physiological functions. In fact, it was the recent recognition of a nitrogen cycle involving nitrate, nitrite and nitric oxide that has directed the research on the role of nitrate (and nitrite) in physiological functions that are known to be regulated by NO (Lundberg *et al.*, 2008).

1.2. The nitrate – nitrite – nitric oxide pathway

Nitric oxide is synthesized in animal tissues from the amino acid L-arginine and molecular O₂ by a family of enzymes, the nitric oxide synthases (NOS), through the L-arginine-nitric oxide pathway. It is produced in the vascular endothelium to regulate blood pressure and acts in the central nervous system as a neurotransmitter (Moncada & Higgs, 1993). Recently, however, it has been shown that nitric oxide production can occur independently of the L-arginine-NOS pathway by reduction of nitrate into nitric oxide, which was only proven in mammals (Jansson *et al.*, 2008).

The nitric oxide synthesized by the L-arginine-NOS pathway can afterwards be stabilized in the blood and tissue by oxidation to nitrite and nitrate, which can then be considered endocrine molecules and have the potential to be reduced back into NO under physiological or pathological conditions. Curiously, the L-arginine-NOS pathway is oxygen dependent while, contrarily, the nitrate-nitrite-NO pathway is activated in situations of ischemia or hypoxia (Lundberg & Weitzberg, 2005), functioning as a back-up system to

guarantee sufficient NO formation when oxygen supply is limited (Lundberg *et al.*, 2008). Furthermore, this alternative nitric oxide production can also occur directly from ingested nitrate. Dietary nitrate is quickly absorbed into the blood stream at the upper gastrointestinal tract, where it mixes with the nitrate formed by oxidation of the endogenous NO synthesized from L-arginine. Although most of the nitrate is excreted in urine, up to 25% is taken up by the salivary glands and then concentrated up to 20-fold in saliva (Lundberg & Govoni, 2004). Once in the mouth, nitrate is reduced to nitrite by commensal bacteria. When saliva enters the acidic stomach much of the nitrite is reduced to form nitrous acid that decomposes further to form NO and other nitrogen oxides (Duncan *et al.*, 1995). Nitrite reduction to NO is enhanced by the presence of reducing compounds such as vitamin C and polyphenols (Peri *et al.*, 2005), which are abundant components of the human diet.

1.3. Physiological roles of nitric oxide

Nitric oxide is known to have multiple health benefits, such as the regulation of the human vasculature; NO is synthesized in the vascular system to achieve vasodilatation, blood pressure regulation, inhibition of endothelial inflammatory cell recruitment and platelet aggregation (Lundberg *et al.*, 2008). In the central nervous system, nitric oxide is a neurotransmitter responsible for several functions including the formation of memory. In the peripheral nervous system, non-adrenergic and non-cholinergic nerves operate through a nitric oxide dependent mechanism to mediate some forms of neurogenic vasodilatation and regulate various gastrointestinal, respiratory, and genitourinary tract functions. Furthermore, it has been showed that some diseases are apparently related to faulty generation or action of NO while on the other hand its production is enhanced during host defense and immunologic reactions (Moncada & Higgs, 1993). In fish NO is also known to have an important role in functions of the brain, neurons, in cardiovascular events, immunological defense and in their general development (Eddy, 2005).

Finally, nitric oxide is also known for having influence in a large range of reproductive functions in animals, particularly in some mammals. Several recent studies show that NO is an important mediator of the hypothalamic-pituitary-gonadal axis, implicated in the control of gonadotrophin secretion, luteinizing hormone surge mechanism, sexual behavior, estradiol synthesis, follicle survival and ovulation (Dixit & Parvizi, 2001). In the brain, NO activates the releasing of the luteinizing-hormone-releasing hormone which reaches the pituitary gland and activates the release of gonadotropins. In the gonads, it plays an important role in inducing ovulation and in causing luteolysis, while in the reproductive tract it is involved in relaxing and constricting the uterine muscle (McCann *et al.*, 1999). Furthermore, it has been shown that nitric oxide plays an important role in sperm performance of fish, enhancing

motility and fertilizing ability (Barman *et al.*, 2012). Still regarding reproduction, nitrate has been suggested as a possible environmental endocrine disruptor, altering the steroidogenesis in vertebrates (Guillette & Edwards, 2005). Since both stimulatory and repressive roles of NO on steroidogenesis have been described, proposed mechanisms for such endocrine disruption involve nitrate to NO conversion in mitochondria, the site of initial steroid synthesis or NO binding to the heme region of P450 enzymes associated with steroidogenesis, impairing enzymes activity.

2. Goals, justifications and general organization of the experimental work

While nitrate ingestion has been vastly studied in mammals, very few attention has been dedicated to this topic in fish (European Food Safety Authority, 2009). Since there is still little knowledge about fish intestinal bacteria (Grosell *et al.*, 2010) and bacteria are responsible in mammals for reducing nitrate into nitric oxide, the same may not occur in fish. Still, if the same effects of nitrate consumption were confirmed in fish, the addition of nitrate to fish diets could be beneficially applied to aquaculture. The oxygen sparing effect of nitrate observed in humans, if confirmed in fish, could be helpful in intensive fish farming, where dissolved O₂ is usually a limiting factor to increased production. Also, the reported ability of nitrate to maximize the energetic power obtained by oxygen consumed could result in increased fish growth if nitrate is fed during growth phase.

Bearing that in mind, this study was developed with the purpose of assessing if chronic nitrate consumption has any growth improvement in fish, as well as if the same O₂ sparing effect of nitrate dietary supplementation observed in mammals can be confirmed in fish. Moreover, due to the reports about nitric oxide influence on several reproductive functions in animals and on the eventual role of nitrate as an endocrine disruptor, we also aimed to study the effect of nitrate dietary supplementation on fish reproductive performance, namely on sex differentiation and egg production and fertilization.

Since zebrafish (*Danio rerio*) has been worldwide accepted as a new physiological model, it was chosen as a fish model for this study. This fish, besides being well known in the aquarist community, has recently gained a particular interest when it comes to scientific research, being used as an experimental model in numerous areas, namely embryology and genetics (Grunwald & Eisen, 2002; Dahm & Geisler, 2006). The great potential of zebrafish as a research model relies on a number of well recognized features. Zebrafish are small and resilient, and don't require much space or an elaborate maintenance (Bilotta *et al.*, 1999). Breeding zebrafish in the laboratory is very easy and genders can be visually distinguished, since males are usually slender and females are rounded at the ventral side (Figure 1). Zebrafish eggs are completely transparent, allowing an easy observation of embryo development (Best *et al.*, 2010). Zebrafish have a short life cycle, achieving sexual maturation in about 3 months after fertilization, and can produce eggs in a daily basis. This makes zebrafish a perfect candidate for the present study for the possibility of using on a

growth trial the same individuals that would be later evaluated for their reproductive performance.



Figure 1 Trio of zebrafish (*Danio rerio*); male on top and two females on the bottom.

Since this study comprises three different goals, it was divided into three trials that occurred in consecutive phases: 1 – growth trial; 2 – reproductive performance trial; and 3 – oxygen consumption trial. This phasing allowed that the same individuals would be used for the entire study, which enabled a better evaluation of chronic nitrate consumption. Also, considering the small size of zebrafish, the time in which the growth and reproductive performance trials took place permitted fish to grow into a more reasonable size to evaluate their oxygen consumption.

To our knowledge, no studies have been published until now about possible effects of dietary nitrate on fish. Therefore, the sodium nitrate (as nitrate source) dietary supplementation levels selected for our study (1, 2, 4 and 8% of the diet) were based on values available in the few studies that served as guideline for the WHO to regulate the acceptable daily intake of nitrate for humans in 1962 (Katan, 2009). These studies reported long term experiments with rats and dogs, in which a maximum of 10% of sodium nitrate was incorporated in diets and no significant effects were observed. Since there was no literature available on this matter on fish, we decided to test sodium nitrate incorporation levels between 1 and 8%, staying beneath the maximum level tested on rats in the mentioned experiments.

3. Materials and Methods

3.1. Experimental diets

Five experimental diets were formulated (Table 1) with different levels of nitrate supplementation in the form of sodium nitrate: a control diet without supplementation (N0) and four diets supplemented at 1% (N1), 2% (N2), 4% (N4) and 8% (N8) of sodium nitrate. Diets were formulated to contain protein and lipid concentrations similar to those present in the commercial diet typically used in zebrafish rearing.

Table 1 Ingredients and proximate chemical analysis of the experimental diets.

Ingredients (% dry weight)	N0	N1	N2	N4	N8
Fish meal ^a	73.6	73.6	73.6	73.6	73.6
CPSP ^b	3.4	3.4	3.4	3.4	3.4
Mineral premix ^c	1	1	1	1	1
Vitamin premix ^d	0.5	0.5	0.5	0.5	0.5
Choline chloride	0.5	0.5	0.5	0.5	0.5
Binder ^e	1	1	1	1	1
Cod liver oil	1.4	1.4	1.4	1.4	1.4
Dextrin	18.7	17.7	16.7	14.7	10.7
Sodium nitrate ^g	0	1	2	4	8
Composition					
Moisture	5.9	6.1	6.1	5.9	5.7
Ash	16.6	17.4	18.2	19.8	22.3
Crude protein	55.1	55.8	56.0	56.6	58.6
Crude lipid	7.7	8.1	8.0	8.9	8.9

^a Steam dried fishmeal. Pesquera Centinela, Peru (crude protein: 71.5% dry matter; gross lipids: 9.5% dry matter).

^b Soluble fish protein concentrate. Sopropeche, France (crude protein: 75.0% dry matter; gross lipids 18.0% dry matter).

^c Minerals (mg kg⁻¹ diet): cobalt sulfate, 1.91; copper sulfate, 19.6; iron sulfate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 8.02 (g kg⁻¹ diet); potassium chloride, 1.15 (g kg⁻¹ diet); sodium chloride, 0.44 (g kg⁻¹ diet).

^d Vitamins (mg kg⁻¹ diet): retinol, 18000 (IU kg⁻¹ diet); cholecalciferol, 2000 (IU kg⁻¹ diet); α -tocopherol, 35; menadion, 10; thiamine, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.

^e Aquacube. Agil, UK.

^g Merck, 7631-99-4. Germany.

For the production of the experimental diets all powdered ingredients were well mixed and then oil and water were added to form a moist blend. The blend was pelleted with a grinder and pellets were dried in an oven at 40°C for 24h. The dried pellets were crushed and

sieved through a battery of sieves with appropriate meshes to obtain feed particles of 400-600 and 600-1000 μm diameter.

3.2. Rearing system and general experimental conditions

All trials took place in a laboratory at the Department of Biology - Faculty of Sciences, University of Porto, using a recirculating aquatic system (Figure 2) based on the one developed by Charlon & Bergot (1984). The recirculating system has a capacity for twenty-one small experimental units (plastic tanks up to 10L volume) through which water continuously flow at a controlled rate.



Figure 2 Recirculating system used in the experiment.

Water quality in the system is assured by means of a biofilter, allowing maintaining ammonia and nitrites at residual levels. A partial water exchange was carried out daily, resulting from the addition of new water (tap water previously dechlorinated and at the same temperature as in the system) to replace that lost by evaporation and during tank cleaning operation. Light period was set to 14h and temperature was maintained at $28^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

3.3. Obtaining zebrafish juveniles for the experimental trials

Eggs of zebrafish were obtained from a broodstock in the bioterium of CIIMAR (Interdisciplinary Centre of Marine and Environmental Research). On the 3rd day post-fertilization, the yolk-sac larvae were transferred to our laboratory. After yolk-sac resorption, on the 5th day post-fertilization, groups of 28-29 larvae were distributed by 18 tanks in the rearing system. Larvae were fed with brine shrimp (*Artemia sp.*) nauplii twice a day (in the morning and afternoon), 6 days a week, for about 8 weeks. Brine shrimp nauplii were

obtained daily by hatching cysts in artificial saltwater, with continuous aeration and illumination for about 24h. Brine shrimp cysts were previously decapsulated as in Sorgeloos *et al.* (1977). During this growth period, larvae were transferred to clean tanks every morning, as described in Charlon & Bergot (1984). Eight weeks after the start of feeding, fish reached an individual weight of about 100mg, the required juvenile size for the experiments.

3.4. Growth trial

In order to evaluate the effect of dietary nitrate supplementation on juvenile growth, five triplicate groups of 20 zebrafish (107.2 ± 0.4 mg individual weight) were randomly distributed by fifteen 5L tanks in the rearing system and each group was fed one of five different diets supplemented with graded levels of nitrate (diets N0, N1, N2, N4 and N8, with 0, 1, 2, 4 and 8% sodium nitrate, respectively, Table 1).

The trial lasted 8 weeks, during which fish were fed by hand to apparent visual satiety twice daily (in the morning and afternoon), 6 times a week. Food was supplied at a particle size of 400-600 μ m during the first three weeks and 600-1000 μ m thereafter to best fit the fish mouth opening. For each experimental group, the food container was weighed at the beginning and at the end of every week to keep track of the amount of food supplied.

Tanks with fish had nylon mesh windows and were placed inside larger tanks (10L volume), facilitating the transference of fish to a clean tank (Charlon & Bergot, 1984), which was done every other day. In each tank, fish were bulk weighed every other week. For that, all fish in the tank were collected with a hand net and excess water was removed with a paper towel; fish were then placed on a water container previously placed on an electronic scale and weighed to the nearest 0.01g. Dead fish were removed from tanks and daily mortality was registered.

3.5. Reproductive performance trial

After completion of the growth trial, fish gender was assessed by the traditional visual examination based on the belly shape and coloration, and four replicates of two females and two males (breeding group) were formed for each original dietary treatment. Three of replicates contained fish only from each of the original replicates, while a fourth replicate contained fish from several of the original replicates. Each breeding group was housed in a 4L-breeding tank – a plastic tank with a plastic mesh lid placed about 1cm above the tank bottom, with some glass marbles as substrate for spawning (Figure 3) – assembled in the rearing system.

The reproductive performance was monitored over five consecutive days. Since zebrafish spawns at dawn (Bilotta *et al.*, 1999), every morning, about 1h-1h30min after the onset of the light period, the mesh lid of the breeding tanks was removed and tanks were inspected for the presence of eggs. Each spawning was registered and eggs were collected by suction with a plastic pipette, cleaned with water from the system and preserved in 70% ethanol after removing excess water (Figure 4). Fish were fed the respective experimental diet in the morning, after egg inspection/collection, and in the evening, before the mesh lid is put back in the breeding tank for the next spawning. For practical reasons, only one set comprising a replicate of each dietary treatment was monitored at a time, and therefore the whole experimental procedure was repeated four times.



Figure 3 Breeding tank ready for spawning. After spawning and fertilization, eggs drop into the space between the net and the bottom of the tank.

At the end of each breeding period, the gender of every fish in all breeding groups was reconfirmed, now using the method described by Yossa *et al.* (2013): fish were firstly anesthetized (by gradual transfer to water at 10°C) and then examined under a stereomicroscope for the presence (in females) or absence (in males) of a prominent genital papilla. All identified females were individually weighed on an electronic scale to the nearest 0.01g, so the number of spawned eggs could be expressed relative to body weight. All the remaining fish of the original tanks used in the growth trial were also analyzed for gender confirmation as described above, in order to evaluate if the dietary treatment had influence on sex ratio.

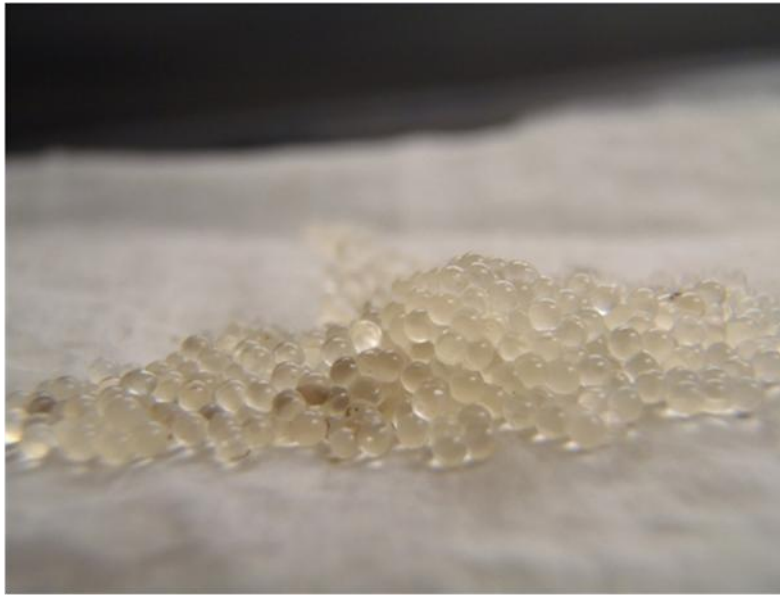


Figure 4 Zebrafish eggs ($\pm 0.7\text{mm}$ diameter) before preservation in ethanol solution.

Ethanol-preserved eggs were later counted and analyzed at the stereomicroscope for the occurrence of fertilization. Fertilization can be confirmed by the presence of a transparent perivitelline space between the embryo (opaque and white in preserved samples) and the egg chorion, since this space is not present in unfertilized eggs. Collected data were used for calculation of fecundity and fertilization rate.

3.6. Oxygen consumption trial

In this trial, only males were used to prevent possible undesirable effects in oxygen consumption due to egg production by females and/or interaction between females and males. From all male fish of each original dietary treatment used in the growth trial, three replicate groups of 13 males were formed to assess the effect of nitrate dietary supplementation on oxygen consumption.

Fish of each group were bulk weighed and housed in 4L-plastic tanks placed in the rearing system. Water flow through the tanks was reduced to about 0.7 L/h, low enough to detect measureable differences in the oxygen concentration but high enough to provide adequate levels of oxygen for fish, as revealed in a preliminary test. One tank without fish was used as a blank. To prevent gas exchange between the tanks and the surrounding atmosphere, Styrofoam lids were placed in every tank.

Fish were fasted for about 48h prior the start of oxygen measurements to reduce the metabolic rate to the basal level. Fish were then fed an amount of food equivalent to 3% of

their body weight, at once. Oxygen concentration was measured in the water inside the tanks with a luminescent dissolved oxygen (LDO) sensor (Hach-Lange LDO 10101) connected to a digital multi-meter (Hach-Lange HQ40D), to the nearest 0.01mg/L (0.1% dissolved oxygen saturation). Measurements were made before feeding to determinate the basal consumption and repeated every 30 minutes the first two hours after feeding, every hour the next three hours, every two hours the next eight hours and less frequently thereafter, in a 24h-cycle.

Oxygen consumption was calculated by the following formula suggested by Kaushik (1980):

$$O = \frac{(S_2 - E_2) - (S_1 - E_1)}{P} \times V + \frac{(S_2 - E_2) + (S_1 - E_1)}{2 \times P} \times D$$

where: O – oxygen consumption (mg/kg body weight/hour); S₁ and S₂ – oxygen concentration in the tank with fish at the periods 1 and 2, respectively (mg/L); E₁ and E₂ – oxygen concentration in the tank without fish at the periods 1 and 2, respectively (mg/L); P – weight of the fish group (kg); V – water volume in the tank (L); D – water flow in the tank (L/h).

3.7. Chemical analyses

Chemical analyses of the diets were performed according to the following procedures: moisture by drying samples in an oven at 105°C until constant weight; ashes by incineration in a muffle furnace at 450°C for 16h; crude protein ($N \times 6.25$) by determination of total nitrogen by the Kjeldahl method using a Kjeltex System 1002; crude lipids by the Soxhlet method, after petroleum ether extraction in a Soxtec System HT.

3.8. Statistic analysis

The homogeneity of variances was verified with the Levene test. Statistical analysis of data was performed by one-way analysis of variance, and means were compared by the Duncan test every time the differences were significant ($P < 0.05$). Analysis was done with the IBM SPSS Statistics 21 software.

4. Results and discussion

4.1. Growth trial

Fish accepted well all the experimental diets and, in general, exhibited a remarkable growth performance (Table 2).

Table 2 Growth performance and feed utilization efficiency of zebrafish fed the experimental diets (N0 – control; N1, N2, N4 and N8 – 1, 2, 4 and 8% nitrate, respectively).¹

Diets	N0	N1	N2	N4	N8
Initial body weight (mg)	107.5 ± 0.5	107.3 ± 1.0	106.5 ± 0.5	107.5 ± 0.5	107.3 ± 0.3
Final body weight (mg)					
Week 2	226.8 ± 6.0 ^a	223.3 ± 15.8 ^a	214.4 ± 6.2 ^{ab}	199.7 ± 3.8 ^{bc}	190.3 ± 6.2 ^c
Week 4	332.0 ± 19.1 ^a	329.8 ± 30.9 ^a	312.1 ± 7.8 ^{ab}	278.8 ± 13.5 ^{bc}	269.3 ± 25.7 ^c
Week 6	438.8 ± 43.3 ^a	442.6 ± 42.2 ^a	408.1 ± 12.7 ^{ab}	373.9 ± 7.7 ^b	372.3 ± 26.4 ^b
Week 8	514.5 ± 62.5	524.1 ± 62.6	475.9 ± 15.8	440.9 ± 9.5	442.3 ± 41.4
Weight gain (mg g ABW ⁻¹ day ⁻¹)					
Week 0-2	51.0 ± 1.9 ^a	50.0 ± 3.9 ^a	48.0 ± 1.6 ^a	42.9 ± 1.4 ^b	39.8 ± 2.3 ^b
Week 2-4	26.8 ± 2.2	27.4 ± 1.9	26.5 ± 0.4	23.6 ± 2.2	24.4 ± 4.5
Week 4-6	19.6 ± 2.8	20.8 ± 0.5	19.0 ± 0.5	20.8 ± 2.7	23.0 ± 3.0
Week 6-8	11.2 ± 1.6	11.9 ± 1.9	11.0 ± 0.2	11.7 ± 1.4	12.2 ± 1.6
Total ²	23.3 ± 1.2	23.5 ± 1.1	22.6 ± 0.3	21.7 ± 0.3	21.7 ± 1.1
Daily growth index ³ (%)					
Week 0-2	9.6 ± 0.4 ^a	9.4 ± 0.9 ^a	8.9 ± 0.4 ^a	7.8 ± 0.3 ^b	7.1 ± 0.5 ^b
Week 2-4	5.9 ± 0.6	6.0 ± 0.6	5.7 ± 0.1	4.9 ± 0.5	5.0 ± 1.0
Week 4-6	4.8 ± 0.8	5.1 ± 0.2	4.5 ± 0.2	4.8 ± 0.6	5.3 ± 0.6
Week 6-8	2.9 ± 0.5	3.1 ± 0.6	2.8 ± 0.1	2.9 ± 0.3	3.0 ± 0.5
Total ²	5.8 ± 0.6	5.9 ± 0.5	5.5 ± 0.1	5.1 ± 0.1	5.1 ± 0.4
Feed intake (mg g ABW ⁻¹ day ⁻¹)					
Week 0-2	57.0 ± 2.9	55.2 ± 1.2	54.4 ± 1.7	52.2 ± 2.4	53.7 ± 3.4
Week 2-4	49.9 ± 1.1 ^a	47.9 ± 1.5 ^a	48.7 ± 2.1 ^a	49.9 ± 1.3 ^a	53.3 ± 1.3 ^b
Week 4-6	51.6 ± 2.5	49.8 ± 5.1	50.3 ± 2.6	53.9 ± 2.9	55.5 ± 4.4
Week 6-8	35.2 ± 1.8	34.7 ± 1.7	34.5 ± 1.6	37.2 ± 0.5	37.8 ± 2.7
Total ²	48.4 ± 1.8	46.3 ± 2.9	47.1 ± 2.0	48.0 ± 0.2	48.6 ± 1.9
Feed efficiency ⁴					
Week 0-2	0.89 ± 0.03 ^a	0.91 ± 0.05 ^a	0.88 ± 0.05 ^a	0.82 ± 0.06 ^a	0.74 ± 0.01 ^b
Week 2-4	0.54 ± 0.06	0.57 ± 0.06	0.55 ± 0.02	0.47 ± 0.03	0.46 ± 0.09
Week 4-6	0.38 ± 0.05	0.42 ± 0.04	0.38 ± 0.03	0.39 ± 0.03	0.41 ± 0.02
Week 6-8	0.32 ± 0.06	0.35 ± 0.07	0.32 ± 0.02	0.32 ± 0.03	0.33 ± 0.07
Total ²	0.48 ± 0.04	0.51 ± 0.05	0.48 ± 0.03	0.45 ± 0.01	0.45 ± 0.04
Survival (%)	100.0 ± 0.0	100.0 ± 0.0	96.7 ± 5.8	91.7 ± 2.9	83.3 ± 15.3

Means in same row sharing a common superscript letter are not statistically different (P>0.05)

[‡] ABW: Average Body Weight = (initial body weight + final body weight) / 2

¹ Values presented as mean ± standard deviation

² Total refers to the values from initial to the final week (weeks 0-8)

³ DGI: Daily Growth Index = (final body weight^{1/3} - initial body weight^{1/3}) / time in days) x 100

⁴ FE: Feed Efficiency = weight gain / feed intake

There was some mortality in groups fed the three diets with the highest nitrate concentration. Although not significantly different among groups, survival was negatively correlated with the dietary nitrate level, which can suggest some toxicity of nitrate for dietary concentrations above the lowest supplementation level used. Moreover, as mortality occurred only during the first experimental weeks and then stopped, older fish seem to be more resistant to presumable toxicity.

Dietary nitrate supplementation did not produce any improvement in zebrafish growth. However, while supplementation levels up to 2% of sodium nitrate also didn't show any adverse effect, higher levels (4 and 8%) caused a decreased growth performance particularly during the first two weeks, as indicated by the lower final body weight, weight gain and daily growth index at that period with diets N4 and N8. By the end of the second week there was a noticeable negative correlation between all growth performance parameters and dietary nitrate levels, supporting the suggestion of nitrate toxic effect, as previously suspected from the survival results.

The negative effects of higher dietary nitrate levels on growth performance were, however, only significant until the second week. In fact, from second week onwards no significant differences were observed among dietary treatments regarding weight gain, daily growth index or feed efficiency, and significant differences persisting in final body weight by the sixth week seem to be a consequence of the huge differences in the performance occurred in the first two weeks. From weeks four to eight it was even observed a reversed tendency on growth among groups comparing to the previous period, as N4 and N8 groups had slightly, though not significant, higher values for some growth parameters.

The fact that growth in zebrafish is limited to a specific size (Biga & Goetz, 2006) probably explains the absence of significant differences in body weight among groups after the sixth week. Since this trial was conducted over a long period taking into account the life cycle of the species, fish that initially grew faster were already close to achieve their final size by the sixth week, hence reducing their growth rate, whereas fish that initially grew slower (fed diets N4 and N8) were still far from their maximum weight and therefore maintained or slightly increased weight gain, reaching a final weight closer to the others. This represents a handicap of using zebrafish as an experimental model since fish used in aquaculture usually have indeterminate growth (with no fixed size). To overcome this constraint, Biga & Goetz (2006) suggest using giant zebrafish (*Danio aequipinnatus*) as a model instead of *Danio rerio*, since it apparently exhibits an indeterminate growth similar to commercially important fish species.

Results obtained in the present trial are somewhat similar to those of the reports mentioned by Katan (2009), which served as the basis to the WHO guideline for nitrate ingestion by humans. In those reports it was stated that dogs fed with diets containing 2% sodium nitrate for over 100 days did not show adverse effects, and that mice fed for their whole life with diets containing up to 10% sodium nitrate exhibited no more than some decreased growth for concentrations above 1% nitrate.

4.2. Reproductive performance

The sex ratio (as percentage of females) and the results obtained in the reproductive performance trial are shown in Table 3.

Table 3 Reproductive performance and female proportion of zebrafish fed the experimental diets (N0 – control; N1, N2, N4 and N8 – 1, 2, 4 and 8% nitrate, respectively).¹

Diets	N0	N1	N2	N4	N8
Total no. of eggs	1299.0 ± 368.4	1508.5 ± 235.0	1124.7 ± 76.9	1096.3 ± 172.8	856.0 ± 538.9
Spawning day ⁻¹	0.7 ± 0.2 ^{bc}	1.0 ± 0.0 ^a	0.8 ± 0.2 ^{ab}	0.9 ± 0.1 ^{ab}	0.5 ± 0.2 ^c
Fertilization (%)	95.4 ± 5.6	97.6 ± 1.0	96.0 ± 2.6	98.2 ± 0.6	97.5 ± 1.7
Eggs spawning ⁻¹	401.2 ± 64.1	301.7 ± 47.0	290.7 ± 57.4	236.6 ± 38.4	306.8 ± 142.2
Eggs BW ^{-1♀}	732.1 ± 195.8	939.5 ± 71.6	801.3 ± 63.5	837.8 ± 163.4	638.7 ± 393.8
Females (%)	16.7 ± 12.6	29.3 ± 7.4	11.0 ± 5.6	17.7 ± 7.0	17.7 ± 9.9

Means in same row sharing a common superscript letter are not statistically different (P>0.05).

♀ Body weight of the females used in the spawning.

¹ Values presented as mean ± standard deviation.

Fertilization rate was above 95% and statistically similar for all the diets. These results apparently contradict previous findings by Barman *et al.* (2012) which suggested that nitric oxide improves fertilization in fish.

Fish fed diets supplemented with sodium nitrate at 1, 2 and 4% showed significantly higher spawning frequency than those fed the diet supplemented with the highest nitrate level. Again, this result suggests toxicity of nitrate above a certain dietary level, now affecting the reproductive performance. Moreover, and interestingly, spawning frequency was significantly increased in fish fed the diet containing 1% sodium nitrate, comparatively to the control. In fish fed the diet containing 1% sodium nitrate spawning occurred in each of the five consecutive experimental days in all the four replicates. Having in mind the positive effects of NO in animal reproduction, we think this finding deserves further research. For the remaining parameters no statistical differences were found.

4.3. Oxygen consumption

Oxygen consumption by zebrafish fed the experimental diets is showed in Figure 5. The control group and the group fed the diet with the highest nitrate supplementation registered the highest oxygen consumption, in both cases significantly higher than that of groups fed diets with intermediate nitrate supplementation (N2 and N4). The overall results clearly point out for an optimum dietary nitrate level leading to a lower oxygen consumption.

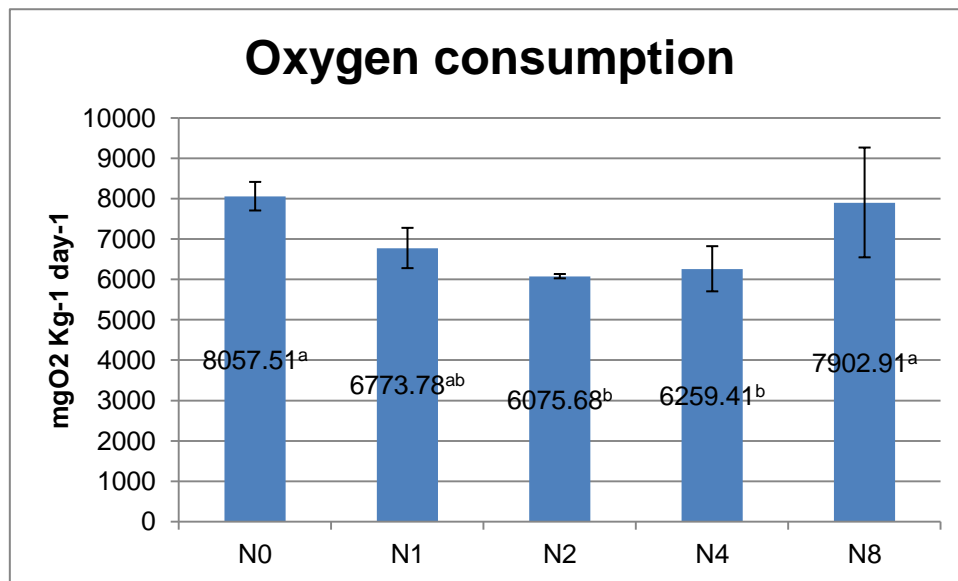


Figure 5 Oxygen consumption of zebrafish fed the experimental diets (N0 – control; N1, N2, N4 and N8 – 1, 2, 4 and 8% nitrate, respectively). Values sharing a common superscript letter are not statistically different ($P>0.05$).

This result corroborates previous studies showing that nitrate ingestion reduces oxygen consumption in humans, having an oxygen sparing effect (Larsen *et al.*, 2007; Bailey *et al.*, 2009; Bescos *et al.*, 2010; Larsen *et al.*, 2010; Lansley *et al.*, 2011b). As far as we know, this effect has not been yet reported in non-humans, and surely not in fish. Although the presence of a nitrate-nitrite-nitric oxide pathway is not confirmed in non-mammals, and therefore in fish, our work demonstrates that the ingestion of nitrate in small percentages significantly reduces the oxygen consumption by zebrafish. Thus, the incorporation of appropriate levels of nitrate in aquaculture fish diets could be useful in situations where dissolved oxygen can be a limiting factor, such as when fish are reared in recirculating systems and/or at high densities.

5. Conclusions

To evaluate the effects of nitrate ingestion on fish growth, reproductive performance and oxygen consumption, four diets with graded levels of sodium nitrate supplementation (1, 2, 4 and 8%) and a control diet were tested on zebrafish.

Dietary nitrate supplementation showed no improvement in zebrafish growth, but at higher supplementation levels (4 and 8% of sodium nitrate) led to a decreased growth performance, which indicates a toxic effect of nitrate at these levels.

Regarding the reproductive performance, only the spawning frequency was affected by dietary nitrate level. Thus, nitrate affected spawning frequency positively at a low level (1% sodium nitrate) but showed toxic effects at a high level (8% sodium nitrate).

Oxygen consumption was lower in fish fed diets with intermediate nitrate levels (2 and 4% sodium nitrate). Results corroborate previous findings that dietary nitrate has an oxygen sparing effect, and broadening this effect to fish, which, to our knowledge, has not been reported yet.

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