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The impact of diet on egg, sperm, and larval quality in a spotted wolffish (*Anarhichas minor,* Olafsen 1772) broodstock.

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Terese Vollstad-Giæver

Abstract

The spotted wolffish (*Anarhichas minor*, Olafsen 1772) has been considered as a highly attractive candidate for aquaculture since the 1990s. Low survival throughout the earliest life stages is, however, restraining the industry from reaching stable commercial levels. Broodstock diet is identified as a key factor for gamete quality. No species-specific broodstock diet has yet been developed for the spotted wolffish. A low-fat diet (LFD) with highly digestible protein sources was formulated to investigate the impact on broodstock growth and fecundity, egg, sperm, and larval quality, using a standard high fat marine fish diet (HFD) as a control treatment. A pre-experimental feeding period of 2 years prior to spawning was carried out. Mean weight and condition factor was significantly reduced in fish receiving the LFD treatment. Total fecundity and egg size did not vary between treatments, but there was a trend for females in the LFD to produce lower amounts of bigger eggs. Fertilization rates were high in both groups and did not differ significantly. Sperm quality determinants did not differ significantly between treatments, except seminal fluid pH (lower in sperm samples from the LFD group), and pH was shown to be positively correlated to sperm curvilinear velocity (VCL). Eggs from the LFD group displayed significantly higher survival from incubation to one week prior to hatching. Fatty acid profiles of unfertilized eggs differed significantly between groups, and egg survival was positively correlated to the essential fatty acids 20:5 n-3 eicosatetraenoic acid (EPA), 20:4 n-6 arachidonic acid (AA), and 22:6 n-3 docosahexaenoic acid (DHA), in addition to omega-3 fatty acids, which were all significantly higher in eggs from the LFD group. Negative correlations between egg survival and the EPA:AA ratio and 18:9 n-1 (oleic acid) was detected. Hatching rates, deformity rates, larval survival from day 0-50 post hatch, length at day 0 and condition factor did not differ significantly between groups, but there was a tendency of higher survival and bigger larvae size in larvae from the LFD group.

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1 Introduction

The spotted wolffish (*Anarhichas minor*, Olafsen 1772) has been considered a promising candidate for cold-water aquaculture in Norway, Canada, and Iceland since the 1990s (Foss et al., 2004; Pavlov, 1994; Tilseth, 1990). The species high tolerance to varying farming conditions (i.e., stocking densities, production units, and water depths during on-growth), high growth rates and fillet yields in addition to friendly behavior and apparent low susceptibility to diseases makes it highly suitable for farming purposes (Le Francois et al., 2021a). They are known for their tasty and lean meat, which also has a high market value (Le Francois et al., 2021b). In 1994, the first artificially fertilized spotted wolffish eggs hatched, and during the early 2000s a full production line was established (Foss et al., 2004; Imsland et al., 2006). In recent years, interest has increased, and new farmers are entering the market.

1.1 Biology

The spotted wolffish belongs to the wolffish family (*Anarhichadidae*). The family consists of five demersal species inhabiting the North Atlantic and Pacific Ocean (Le Francois et al., 2010). The two Pacific species are the wolf-eel (*Anarrhichtys ocellatus)* and Bering wolffish (*Anarhichas orientalis*), whilst three species are found in the North Atlantic, namely the common wolffish (*A. lupus*), spotted wolffish and the northern wolffish (*A. denticulatus*). The wolffishes are recognized by their long, muscular bodies and a rounded head with heavy jaws making them well suited to consume hard-bodied prey such as crustaceans, echinoderms, and other marine invertebrates (Le Francois et al., 2021a; Templeman, 1986). The spotted wolffish is an artic-boreal species, mainly distributed in the Barents and North Atlantic Sea (Le Francois et al., 2010) (Figure 1). They are a stenotherm species found at temperatures from -1-7°C, reaching sizes of over 100 cm and weights of more than 20 kilos (Falk-Petersen et al., 2001).

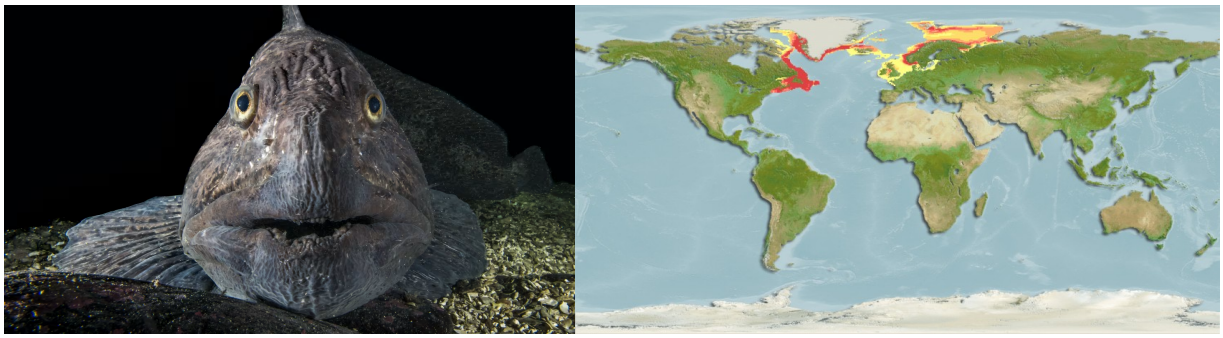

Figure 1. Picture of the spotted wolffish (Photo: Erling Svensen) (left), and geographical distribution of the spotted wolffish (right). Red indicates the highest abundance, decreasing color intensity represents decreasing abundances. (Source: AquaMaps, 2019).

Tilseth (1990) acknowledged the spotted and common wolffish as promising species for cold-water aquaculture. The spotted wolffish, however, excelled the common wolffish due to higher growth rates and fecundity, and a behavior that is more conductive for farming purposes (Moksness & Stefanussen, 1990; Pavlov, 1994; Pavlov & Moksness, 1994a; Pavlov & Moksness, 1994b). In captivity, they exhibit a social, non-aggressive behavior, mostly occupying the bottom in dense clusters (Johannessen et al., 1993) (Figure 2). Despite the great potential for aquaculture, there are few commercial farmers of the spotted wolffish. From 2004-2006, a commercial company (Tomma Marin Fisk AS, Nordland County, Norway) produced 100-120 tons annually. A pump failure resulted in the loss of most of the standing stock and the broodstock and production was thereafter terminated. In 2013 a new company, Aminor AS (Halsa, Nordland County, Norway) established a full production line and are currently producing 50-100 tons annually (Le Francois et al., 2021a). For the spotted wolffish, broodstock management in culture is not properly optimized, resulting in an unstable supply of eggs and sperm, of variable quality (Foss et al., 2004; Le Francois et al., 2021a). This is currently identified as the main biological bottleneck for commercial production of the spotted wolffish (Beirão & Ottesen, 2018).

Figure 2. Spotted wolffish in captivity on Akvaplan-nivas research station FISK (Tromsø, Norway), aggregating in dense clusters (Photo: Akvaplan-niva).

1.2 Reproductive biology

The spotted wolffish is long-lived and reaches first maturation around the age 6-8 years for females and 7-9 years for males (Le Francois et al., 2010). They form monogamous couples 4-5 months prior to spawning (Johannessen et al., 1993). The spotted wolffish are iteroparous determinate spawners, usually spawning once a year after reaching mature age (Johannessen et al., 1993; Pavlov & Moksness, 1996), and fertilization is presumed to be internal (Kime & Tveiten 2002; Le Francois et al., 2007; Pavlov, 1994). They produce large negatively buoyant eggs (5-6.5 mm) of a light-yellow color with a semitransparent eggshell and visible oil-droplets (Falk-Petersen & Hansen, 2003). Eggs are released in a sticky gelatinous ovarian fluid and formed to a ball-like shape by the female. The spotted wolffish display parental care, and the eggs are guarded by the male until hatching (Johannessen et al., 1993). They have a long embryogenesis and incubation time of 800-1000 day-degrees (DD) (Falk-Petersen & Hansen, 2003; Falk-Petersen & Hansen, 2005).

The spotted wolffish display a reproductive biology common for species with direct and early ontogeny (Pavlov & Moksness, 1994a). This is recognized by lower fecundity, bigger egg size and a prolonged growth inside the egg envelope before hatching at an advanced stage of development typical of 'precocial larvae' (Falk-Petersen & Hansen, 2003). Due to the long development inside the egg, the larval morphology at hatch is described as advanced (Falk-Petersen & Hansen, 2003). The newly hatched larvae have a highly functioning digestive system in addition to well-developed teeth, mouth, and jaws, making them suited to commence feeding shortly after hatching (Falk-Petersen & Hansen, 2005; Foss et al., 2004). The yolk sac is mostly absorbed at the point of hatching and is completely absorbed 3-6 weeks post hatching (Falk-Petersen & Hansen, 2003).

Sperm characteristics of the spotted wolffish greatly differ from other teleost species (Beirão & Ottesen, 2018; Kime & Tveiten, 2002; Santana et al., 2020). Males produce sperm which is already motile at stripping (Kime & Tveiten, 2002). Male wolffish have a low gonadosomatic index (Falk-Petersen & Hansen, 1991) and produce small amounts of sperm (Johannessen et al., 1993). Pavlov & Moksness (1996) demonstrated that mature males produced sperm all year, with a peak during the spawning season and a decrease at the end of spawning period. The sperm obtains its motility up to 48 hours after stripping and becomes immotile in contact with saltwater (González-Lopez et al., 2021; Kime & Tveiten, 2002). Sperm cell movement is

described as slow and 'wiggly', which are characteristics associated with internal fertilization (Kime & Tveiten, 2002).

In captivity, their natural spawning behavior is interfered. Therefore, mature males and females must be identified prior to spawning and gametes have to be collected manually. The females release unfertilized eggs, and fertilization must be performed artificially *in vitro* (Berião & Ottesen, 2018; Foss et al., 2004). The males and females often have asynchronous spawning, and both amount and quality of the sperm are limiting factors (Le Francois et al., 2007; Santana et al., 2020). Due to the slow sperm movement, a contact time of 3-9 hours between eggs and sperm is necessary to obtain successful fertilization (Beirão & Ottesen, 2018; Johannessen et al., 1993; Pavlov, 1994).

1.3 Gamete quality

Reproductive fitness in terms of gamete quality is essential for successful reproduction for domestic and wild animals (Reading et al., 2018). Gamete quality is commonly described as the eggs or sperm's ability to support the successful development of viable offspring (Bobe & Labbè, 2010; Migaud et al., 2013). In particular, egg quality is often referred to as the eggs' ability to become fertilized and subsequently develop normally (Bobe, 2018), while sperm quality defines the sperm cells traits to successfully fertilize an egg and enable normal development (Bobe & Labbè, 2010). Poor gamete quality is associated with low fertilization rates, high mortalities, and developmental issues through embryogenesis and even after hatching (Brooks et al., 1997). Availability of high-quality gametes is crucial for the development of new species in aquaculture (Izquierdo et al., 2001). High mortality during the incubation and start-feeding period as a consequence poor egg and sperm quality is currently a major restraint for commercial production of the spotted wolffish (Le Francois et al., 2021a). Tveiten et al., (2004) proposed that survival through the earliest life stages is the main limiting factor for commercial production in aquaculture.

Parameters that affect gamete quality are primarily related to the environmental conditions experienced by the spawning parental fish during maturation, such as temperature, water quality general welfare (Frantzen et al., 2008; Le Francois et al., 2021a; Tveiten et al., 2001; Tveiten et al., 2004). Epigenetic factors are also known to determine quality and survival during the gametogenesis and embryogenesis (Labbé et al., 2017), in addition to other physical factors like the environment the eggs are being incubated in and larval rearing conditions (Hansen & Falk-Petersen, 2002a).

Parentally derived nutrients through broodstock diet have major impact on gamete quality and has been identified as a key for reproductive success (Brooks et al., 1997; Izquierdo et al., 2001; Izquierdo et al., 2015; Reading et al., 2018). An egg is the final product of an oocyte, generated through several stages of the oogenesis (Brooks et al., 1997). Subsequently, a spermatozoa is the final stage of the spermatogenesis. Through the oogenesis and spermatogenesis, the gametes are synthesized from available nutrients derived from the parental diet (Mommens et al., 2015). Such nutrients include lipids, proteins, amino acids, vitamins, and minerals (Reading et al., 2018). Broodstock diet is especially important for egg quality. During the oogenesis and final maturation, reserves obtained from the diet are carried into the oocytes (Brooks et al., 1997). These dietary compounds are necessary as they fulfil the embryos nutritional requirements for development through the embryogenesis and until the larvae can start feeding exogenously (Tveiten et al., 2004). This has been identified as particularly important for species with larger eggs and a long development inside the egg envelope (Fontagnè-Dicharry et al., 2010). Dietary compounds have also demonstrated to impact sperm quality (Butts et al., 2015; Ciereszko & Dabrowski, 1995; Yildiz et al., 2021).

1.4 Gamete quality determinants

Predictive tools for gamete quality may be helpful for assessing viability (Kjørsvik et al., 2003). Poor egg batches are highly time and resource demanding, and early predictive determinants could lead to a better selection of eggs that are more likely to be fertilized and develop normally (Avery et al., 2009). When evaluating egg quality and fertilization rate, survival through the incubation time and hatching success are commonly used, but egg and larval size, early cell morphology, time of hatching, deformity rate and chemical composition of the egg may also be used as descriptive indicators (Bobe & Labbè, 2010; Sink & Lochmann, 2008; Stuart et al., 2020; Tveiten et al., 2001). Total fecundity, the number of eggs produced over a spawning season (Izquierdo et al., 2001), may also be used as a measure of reproductive success in addition to the condition factor (K-factor) of the spawning female.

A successful fertilization is followed by a cortical reaction that activates the egg, and a process of mitotic divisions of cells referred to as blastomeres, normally developing in symmetrical patterns (Avery et al., 2009). Strong correlations between early blastomere morphology and mortality, deformations and hatching rates were proposed by Kjørsvik (1994). Abnormal patterns are described as asymmetrical cells in terms of size and shape, often with poor adhesion between the blastomeres (Shields et al., 1997). Evaluation of the early blastomere is considered

a strong indicator for egg quality in the Atlantic cod (*Gadus morhua*) (Kjørsvik et al., 2003). Tveiten et al., (2001) found correlations between early blastomere cleavages and survival to hatching in common wolffish broodstock exposed to high temperatures during the final maturation, demonstrating that blastomere morphology may be a useful indicator.

Lipids and their fatty acids are critical for several biological functions (Cowey & Sargent, 1977; Tveiten et al., 2004). They are central for development of membrane phospholipids and neural tissue, in addition to cellular and physiological functions (Bell et al., 1997; Betancor et al., 2012; Sargent et al., 1999). Fatty acid profiles of teleost fish eggs are commonly characterized with high levels of omega-3 (n-3) fatty acids, especially 22:6 n-3 docosahexaenoic acid (DHA), 20:5 n-3 eicosatetraenoic acid (EPA), in addition to monounsaturated fatty acids (MUFAs), saturated fatty acids (SFAs), and 20:4 n-6 arachidonic acid (AA) (Almansa et al., 1999; Tveiten et al., 2004). EPA, AA, and DHA are recognized as critical essential fatty acids, with direct impact on reproductive success in terms of fecundity, fertilization rates and survival (Mazorra et al., 2003; Salze et al., 2005; Xu et al., 2019). During the embryogenesis, lipids obtained from the yolk fulfils vital roles (Tveiten et al., 2004). Several studies have found correlations between dietary fatty acids and lipid composition of eggs, suggesting that broodstock diet influences the chemical composition of an egg (Almansa et al., 1999; Morais et al., 2014). Tveiten et al., (2004) characterized spotted wolffish eggs with relatively low lipid content compared to species like the common wolffish and salmonids (*Salmonidae*), with high levels of EPA, DHA, 16:0, 18:1 n-9 (oleic acid), in addition to low ratio between EPA:AA.

Motility, viability, and density are considered to be the most powerful determinants for sperm quality (Beirão & Ottesen, 2018; Gallego et al., 2018; Gonzàlez-Lopez et al., 2021). For density, spermatocrit, the packed layer of cells after centrifugation of a sperm sample, is a commonly used indicator (Santana et al., 2020). Sperm motility is usually evaluated either subjectively or objectively (Gallego et al., 2018). Computer Assisted Semen Analysis (CASA) is an objective tool used to track and quantify sperm quality markers in terms of motility characteristics (Kime et al., 2001). For the spotted wolffish, motility (MOT, %), curvilinear velocity (VCL, μms^{-1}), and beat cross frequency (BCF, H_z) has been identified as the most powerful CASA parameters (Kime & Tveiten, 2002). MOT is a measure on percentwise motile cells in the sample, whilst VCL represents the total distance moved by a sperm cell, divided by the time it takes for the sperm to navigate the entire track (Kime et al., 2001). BCF is the frequency of turning point of the spermatozoa head in cycles and is used as a measure on head

movement (Kime & Tveiten, 2002). Both seminal fluid pH and osmolality have been linked to motility characteristics and is also considered a determinant for sperm quality (Alavi et al., 2005; Beirão et al., 2020; Cosson, 2004; Dreanno et al., 1999).

1.5 Objectives

Nutritional requirements for successful gamete production are species specific. As such, formulated diets used for farming purposes should be developed to meet the nutritional requirements of each species in question (Brooks et al., 1997). No such diet has yet been developed for the spotted wolffish, and the broodstock is currently fed a standard diet for marine fish with a high-fat level and fishmeal as the main protein source.

In the present study, a new diet, similar to the composition of the natural prey of the spotted wolffish was specifically designed, with a low-fat content and highly digestible protein sources. The aim of this study was to investigate the impact of this experimental broodstock diet in comparison with a standard commercial one. The specific research questions were the following ones:

1. Does the use of a specifically developed low-fat diet result in improved broodstock growth, fecundity, and reproductive performance in terms of fertilization rates and gamete quality?
2. Does the use of such a novel broodstock diet improve embryo and larvae survival and quality?

Reproductive performance was evaluated in terms of broodstock total fecundity and growth, egg, sperm, and larval quality. Egg quality was evaluated using egg size, fertilization rates, evaluation of early blastomere morphology, survival throughout the incubation period, and lipid analysis of unfertilized eggs. Sperm quality was evaluated using CASA, spermatocrit, seminal fluid osmolality and pH, whilst larvae quality was determined using hatching rate, deformity rate, survival from day 0-50 post hatch, length at day 0 and length, mean weight, and K-factor at day 50 post hatch.

2 Materials and methods

2.1 Experimental fish and rearing conditions

The experimental fish consisted of 60 individuals from a cultured broodstock of spotted wolffish ranging in size from 3-5 kg. The experimental fish originated from eggs incubated at Akvaplan-nivas research station (FISK, Tromsø, Norway) in December/January 2015, and were subsequently reared at a commercial farm (Aminor AS, Halsa, Nordland County) until Autumn 2020, when 60 individuals were transported to the experimental facilities of Akvaplan-niva. On arrival, the fish were sex differentiated using ultrasound and tagged, and randomly split into 2 groups in replicates (4 tanks) after a period of acclimatization. During the pre-experimental feeding period from March 2021 prior to spawning in January/February 2023, the fish were anaesthetized (FinQuel vet., Sanvacc, Norway, 0.2g l^{-1}), weighed (BW, kg) and length measured (BL, cm) at uneven intervals. Fish condition factor (K) was calculated as: $K=100(WL^{-3})$ where W is body weight (g), and L is the body length (cm). The fish received formaldehyde (36%) treatment twice during the pre-experimental period to prevent parasitic infections. The experimental period lasted from spawning in January/February 2023 until the end of start feeding period in September 2023.

Water was pumped from 60 meters depth, and the experimental broodstock were kept in 1.5 x 1.5 meter 1.75 m^3 fiberglass tanks at ambient sea water temperature (3-9 °C) (Figure 3). Oxygen saturation was >90% throughout the experimental period, while a low light level photoperiod for Tromsø, Norway (69° 45” 33” N, 19° 02 ”46” E) was followed. The fish were hand-fed to satiation 3-4 times a week. Other general husbandry conditions followed standard in-house procedures for wolffish (FISK, Tromsø). Each replicate consisted of 5 males and 10 females. There is no available data on previous spawnings during the pre-experimental period.

Figure 3. Overview of sea water temperature in the experimental facility (FISK, Akvaplan-niva, Tromsø, Norway) during the pre-experimental and experimental period from 2021-2023.

2.2 Diets

Two different diets were used. Diet 1 (HFD) was a high-fat commercial feed for marine fish (Vitalis Prima, Skretting AS, Norway), and Diet 2 (LFD) was a low-fat diet, specially designed by the project group, produced by Sparos I&D (Portugal). The experimental LFD was produced in February 2021 and 2022 and arrived at the research station in March 2021 and 2022. Pellet size for both diets were 13 mm. The protein level in both diets was similar, but the protein sources differed. The commercial HFD had fishmeal as the main protein source, while this was replaced with squid, krill, and mussel meal in the experimental LFD. The fat content in the LFD diet was 12% compared to 18% in the HFD diet. The Vitamin content also differed between the two diets, where the LFD had an almost four times higher content of Vitamin A and D, and slightly higher content of Vitamin C and E. Full recipe and chemical composition of the LFD is given in Appendix 1. The differences between known compounds in both diets are presented in Table 1. Two replicates received the HFD treatment, and two replicates received the LFD treatment.

Table 1. Formulation and chemical composition of the two diets used for this experiment.

Compound	Commercial diet (HFD)	Experimental diet (LFD)
Vitamin C (mg/kg)	1000	1271.5
Vitamin E (mg/kg)	600	700
Vitamin D (IU/kg)	1125	3070
Vitamin A (IU/kg)	7500	31921
Protein (%)	54	55
Lipid (%)	18	12
Ash (%)	10.8	10.9
Fiber (%)	0.4	0.8

2.3 Gamete collection and incubation

Close to spawning in January 2023, the fish were closely monitored several times a day. Females close to spawning were recognized by their bulging abdominal area, where a mirror attached to a stick was used to observe and evaluate the size of the genital pore opening which increased during ovulation (Figure 4). Females ready to be stripped had an opening of 0.5 - 1 cm and displayed spawning behavior such as rolling, laying on the side and vibrating movements. Eggs was collected from the first 5 spawning females in each treatment group.

Figure 4. Spotted wolffish female close to spawning, recognized by the bulging abdomen (left) and monitoring of opening in the genital pore using a mirror attached to a stick (right). (Photo: Terese Vollstad-Giæver).

Fish ready to be stripped were sedated using FinQuel vet., (0.2g l^{-1}) for 5-10 minutes. Prior to stripping, weight was recorded, and the genital area was wiped dry to avoid saltwater contamination. A light pressure was applied to the abdominal area, stroking from front to back, followed by a flow of eggs collected in a beaker. Stripping of males followed the same procedure for sedation. Prior to sperm collection, a light initial pressure was applied to the middle abdominal area to remove urine. A pipette was placed on the urogenital papilla, and sperm was collected by applying pressure to the lateral testis region accompanied with a light suction from the pipette (Figure 5). Both eggs and sperm were kept cold in a fridge at 4 °C from collection until analyzes and fertilization occurred.

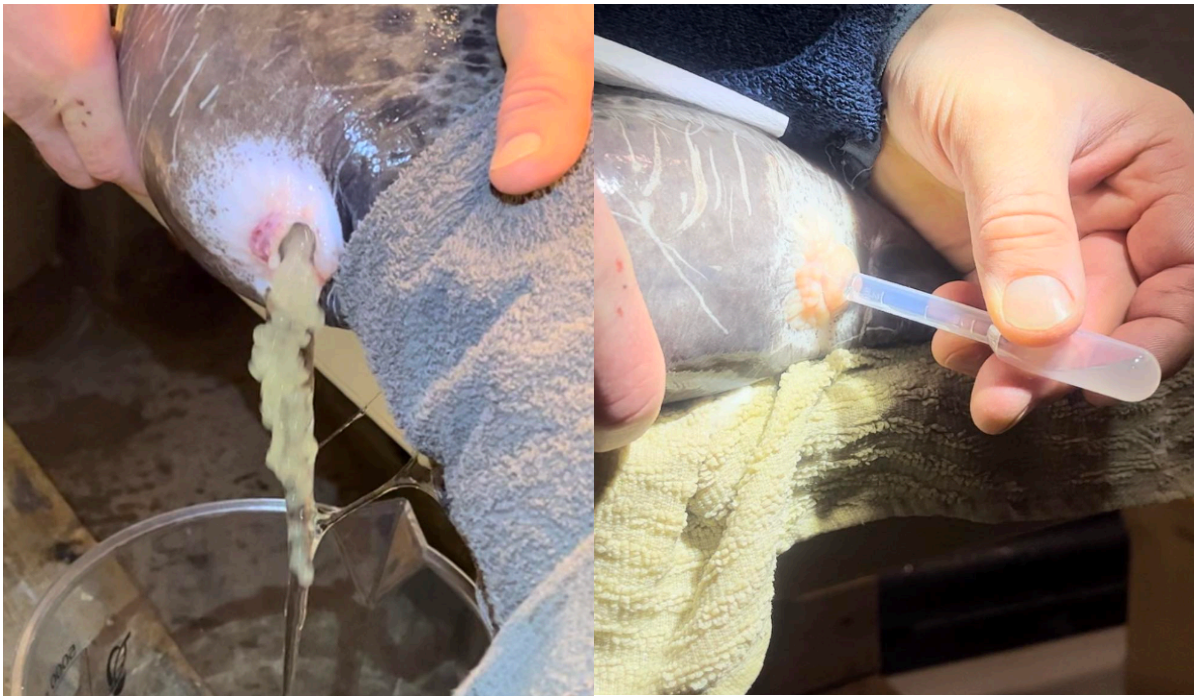

Figure 5. Stripping of spotted wolffish female (left) and male (right). (Photo: Terese Vollstad-Giæver).

Before fertilization, the diameter of 20 eggs were measured using a magnifying glass with a measuring ocular. For measures of total fecundity, approximately 100 ml of eggs and ovarian fluid were collected, and the total number of eggs counted. A sample of 10 g unfertilized eggs was collected from each spawning female and stored in a -80 °C freezer for analysis of fatty acid composition in May 2023.

Eggs from each female were fertilized using the sperm from 2 males from the same experimental group. From each egg batch, 0.15 l of eggs were fertilized using 3 ml of sperm. Since sperm from the spotted wolffish is already motile at stripping, fertilization of eggs followed the procedure of dry fertilization described by Beirão & Ottesen (2018). Eggs and sperm were mixed together by pouring between two beakers 8-10 times, before they were stored in the fridge. Contact time between the eggs and sperm was 3 hours, with additional mixing every hour. The 0.15 l of eggs were incubated in small upstream incubators (12 cm diameter, 20 l h^{-1}) (Figure 6) in triplicates (0.05 l per replicate) after fertilization. However, from the first egg batch from the LFD group, 0.45 l was incubated (0.15 l per replicate). Waterflow in the incubator was stopped before the eggs were poured in, allowing the eggs to sink and stick together. Ovarian fluid was removed using a siphon before the flow was turned on after 15 minutes.

After an initial incubation period of 18-24 hours, the eggs were disinfected using Buffodine (FishTech AS, Norway, 10ml l^{-1}) for 10 minutes. The egg surface disinfectant was mixed in a separate tray where the eggs were treated, and after treatment the eggs were rinsed with saltwater and returned to their incubators. Eggs were disinfected every 14th day until reaching 400 DD. The first removal of dead eggs occurred 14 days after fertilization. Prior to disinfection, dead eggs were removed. After reaching the age of 700 DD, eggs were left undisturbed to avoid early stress-induced hatching. The eggs were incubated under dimmed light (0.01 ± 0.01 flux). Temperature followed ambient seawater temperature (3-8 °C). Survival throughout the incubation period was determined at 400 DD and 1-week pre hatch at 980 DD, as a proportion of number of eggs incubated.

Figure 6. Incubation unit consisting of small upstream incubators (left) and spotted wolffish eggs at the eyed stage at 400 day-degrees (DD) (right). (Photo: Terese Vollstad-Giæver).

2.4 Sperm analysis

2.4.1 Subjective and objective sperm analysis

Immediately following stripping, a subjective sperm analysis was performed under a microscope, evaluating density and motility on a scale from 0-3. Samples that scored 0 or 1 on either of the parameters were discharged. After deciding whether the sperm was of sufficient quality to be used for fertilization, CASA was used to perform an objective sperm analysis according to the procedures described by Santana et al., (2020). The CASA software contains species specific parameters which were adjusted to fit the experimental conditions previously described.

Prior to recording, the sperm was diluted using a commercial extender developed by Kime & Tveiten (2002), consisting of 145 mM NaCl, 4.55 mM $CaCl_2$, 4.83 mM $KHCO_2$, 2.37 mM $MgSO_4$ and 1 mM glucose (Appendix 2). 100 µl of sperm was diluted in 200 µl of commercial extender mixed in an Eppendorf tube. 4 µl of the dilution was pipetted to one chamber, diluting sperm, and extender to 1:3. Each sperm sample was recorded in duplicates, recording each replicate 4 times, tracking different parts of the chamber to obtain a representative view of the sample. Evaluated CASA parameters were sperm motility (MOT), curvilinear velocity (VCL) and beat cross frequency (BCF) after Kime & Tveiten (2002). The video frame per second was set to 25, cell movement below 9 µm was considered to be drifting, and the head area was set to 10-50 mm^2 after Santana et al., (2020).

2.4.2 Spermatocrit and pH

Spermatocrit (%) was analyzed in triplicates for each male. Capillary hematocrit tubes were filled with sperm and closed with a double layer of clay before centrifuged at 8000 G for 10 minutes. Spermatocrit was calculated by measuring the length of the packed cell layer divided by the total volume of fluid in the hematocrit tube x 100. The remaining sperm volume was pipetted to a 2.5 ml Eppendorf tube and centrifuged (8000 G, 10 minutes) to separate the seminal fluid from the sperm cells. The seminal fluid was pipetted to a new Eppendorf tube where pH was measured using pH strips (range 5-9, Hydrion, Sigma-Aldrich) and stored in a -80 °C freezer prior to analysis of seminal fluid osmolality. Seminal fluid osmolality (mOsm/kg) was measured using a Fiske One-Ten Osmometer (Fiske Associates, MA, USA) after calibration with 3 standards (100, 290 and 850 mOsm/kg). Samples from each male were measured three times in technical triplicates.

2.5 Egg analysis

Around 18-24 hours after fertilization, 30 eggs were fixed using a 1:20 (v/v) solution of glacial acetic acid and physiological solution (9% NaCl) to determine fertilization rate and evaluate cell morphology at the 4 blastomere stage. Early cell morphology was evaluated using a dissecting microscope after cell-symmetry and adhesion, and was scored on a scale from 0-3, where 0 represented unfertilized eggs, 1 represented abnormal cleavage where the cells were of different size and had split, 2 represented cleavages where the cells were of asymmetrical size and shape, and 3 represented normal cleaving of symmetrical cells. This was not performed

on the first batch from each group. Fertilization rate was determined as the number of fertilized eggs divided by the total number of evaluated eggs.

2.6 Fatty acid composition and lipid analysis

The unfertilized egg sample was thawed at room temperature for 15 minutes before a subsample of 0.5 g for each egg batch was collected for analysis. Extraction of the lipids was performed following the procedures of Folch et al., (1957) and Tveiten et al., (2004). Abbreviations used are listed in Appendix 3.

The first extraction was performed by adding 3 ml MeOH/BHT, followed by a flushing of the sample using N_2. The sample was then stirred for 10 seconds and placed in a -20 °C freezer for 5 minutes. The extracted sample was passed through a prewashed filter with DCM:MeOH (2:1) to a new tube, before additional DCM:MeOH (2:1) was used to wash the filter. The sample was then stirred for an additional 10 seconds, before passing through the same filter. The sample tube was washed with 5 ml DCM:MeOH (2:1) m/BHT, which was also filtered. After extraction, the samples were washed by addition of 5 ml 0.88% KCl and centrifuged at 2000 rpm for 10 minutes at a temperature of 4 °C to separate the water phase from the organic phase. The water phase was removed from the tube, and the total volume in the tube was reduced to ¼ by blowing with N_2 before the organic phase was transferred to a new tube. Extractable organic matter was determined by damping the organic phase dry using rocket and vacuum desiccator for 30 minutes.

Methylation was performed using 2 ml 1% H_2SO_4 to MeOH, before the samples were flushed using nitrogen and shaken to ensure proper mixing of the sample. The sample was stored in a heating chamber holding 50 °C overnight, before the samples was cooled and 2 ml $KHCO_3$ was added. 5 ml hexan:ether was added before being centrifuged at 1500 rpm for 2 minutes. The organic layer was added to a new tube, before 1 ml hexan per mg lipid was added. The lipids were quantified using GC-FID by addition of 21:0 fatty acid as standard.

2.7 Hatching and start-feeding

Hatching occurred approximately 6 months after incubation (980-1000 DD), lasting from the end of July to mid-August 2023. Larvae that hatched before 900 DD were classified as premature. Hatched and free-swimming larvae were collected and counted individually using a pipette and moved to the start feeding unit. Larvae with visible deformations (twisted and bent

notochords, crippled tails, and bent necks) were counted removed and euthanized with an overdose of anesthetics (FinQuel vet., 2 g l^{-1}). The start feeding unit consisted of 2 downstream raceways (240x40cm) with one separate cage per egg batch (Figure 7). Each cage had a separate water intake (40 l h^{-1}) to ensure similar water quality in all units. Water depth was 15 cm. Oxygen were >90% saturation throughout the start feeding period, and temperature followed ambient sea water temperatures, varying from 6-9 °C. The raceways were cleaned daily.

Each cage had a separate automatic feeder (Eheim, Germany) providing feed 6 times a day, 1 g per feeding in addition to handfeeding twice a day. The larvae were offered food 1 day post hatching and received a mix of 0.5-and 0.8-mm pellet size of Skretting Clean Assist (Skretting AS, Norway) until 14 days post-hatch. For the rest of the start feeding period, the larvae were exclusively fed with 0.8 mm feed. The total number of hatched individuals was determined two weeks after the first egg hatched in each incubator. Hatching rate was determined as a proportion of number of eggs incubated. Dead larvae were registered until day 50 post hatching. A total of 10 larvae were collected from each unit on day 0, anaesthetized (FinQuel vet., 0.1g l^{-1}), length measured to the nearest mm using a ruler and returned to their tanks. At day 50, 10 larvae from each batch were length measured again, and two mean weights were recorded from each experimental unit. Larvae condition factor (K) was calculated as: $K=100(WL^{-3})$ where W is body weight (g), and L is the body length (cm).

Figure 7. Spotted wolffish eggs hatching at 1000 day-degrees (DD) (left) and experimental start feeding unit with separate water intakes and automatic feeders (right). (Photo: Terese Vollstad-Giæver).

2.8 Data analysis

Statistical analysis was carried out using R software (R Core Team, version 2.3.6). Normality was evaluated before the non-parametric Mann Whitney U-test (wilcox code) was used to test for statistically significant differences between the two groups for every parameter evaluated. Data was given as mean ± standard deviation (SD). A threshold of $p<0.05$ was used to determine significant statistical differences between groups. The non-parametric Spearman rank sum test (cor.test, method=spearman code) was used to test for correlations between concentrations of fatty acids, survival throughout the incubation period, hatching rates, and larval performance, in addition to fecundity, egg size and female body weight. The same applied for sperm MOT, VCL, BFC and pH, and spermatocrit and sperm volumes. A threshold of $p<0.05$ determined whether the correlation was significant or not. Estimated rho coefficient (R-value) given between -1 and 1, determined whether the correlation was positive or negative in addition to correlation strength.

2.9 Ethical statement

This study was carried out in accordance with the Norwegian regulations for use of animal experiments. The feed trial was carried out in an approved facility (FISK, Tromsø, 10764, T-T-4400) by the Norwegian Committee on Ethics in Animal Experimentation, issued by the Norwegian Food Safety Authority (Mattilsynet, FOTS). All efforts were made to minimize fish suffering and the project have been performed by trained and licensed personnel.

3 Results

3.1 Broodstock growth and fecundity

Initially, the 5 spawning females in the HFD group were heavier compared to the 5 spawning females in the LFD group. This difference remained throughout the experimental period and was significant from 10th of May 2022 (p=0.02) (Figure 8). Mean weight for all males in each treatment group was significantly lower in the LFD group from 11th of August 2022 (p=0.03) (Figure 9).

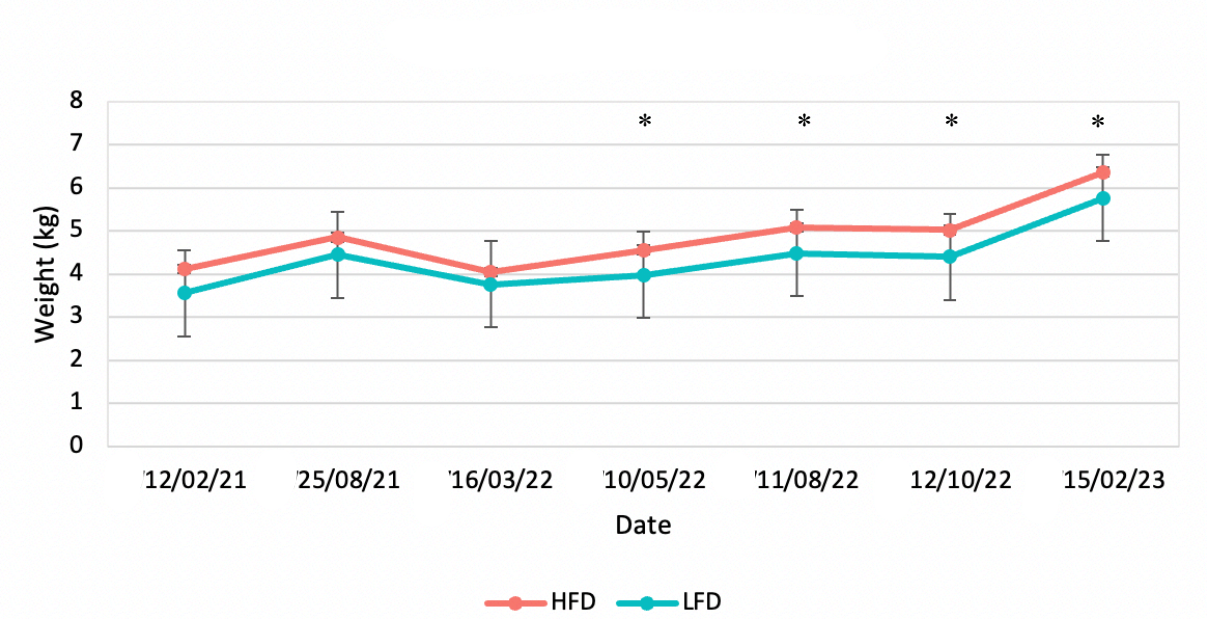

*Figure 8. Mean weight (kg) for the spawning females reared at different dietary treatments (n=10, 5/ group) during the pre-experimental feeding period of two years. Values are given as means ± SD. * Denotes significant differences between treatments (p<0.05).*

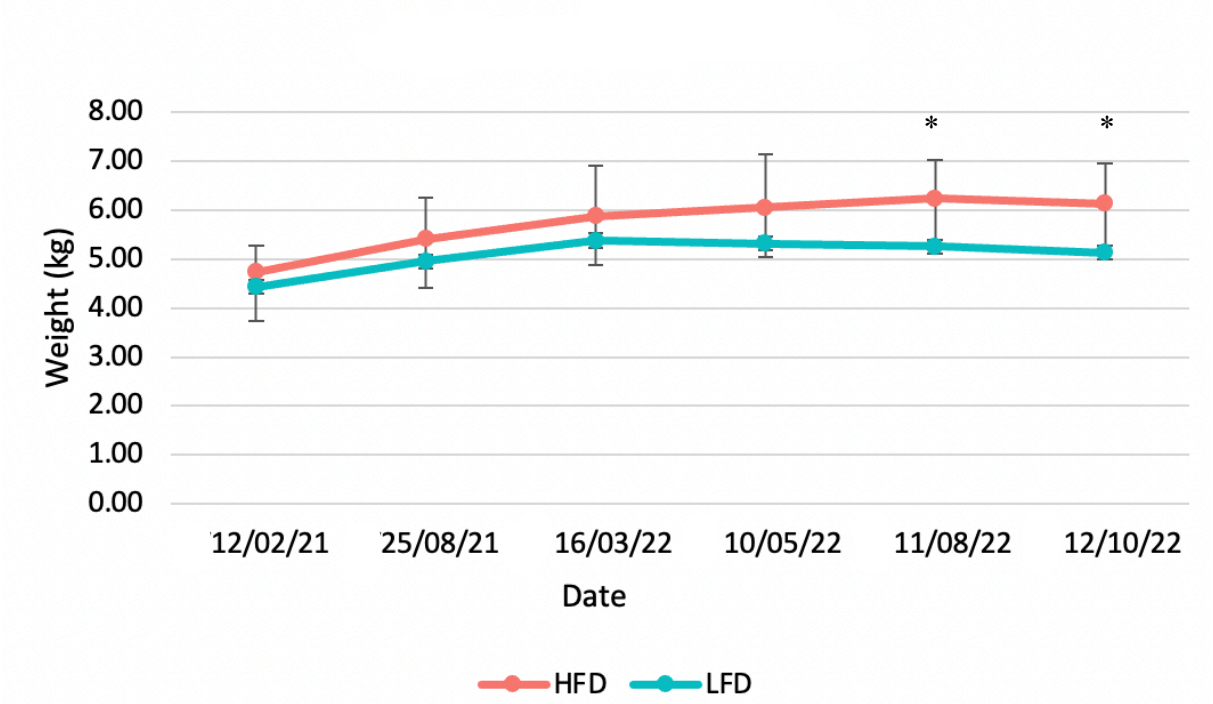

*Figure 9. Mean weight (kg) for all males (n= 20, 10/ group) reared at different dietary treatments during the pre-experimental feeding period of two years. Values are given as means ± SD. * Denotes significant differences between treatments (p<0.05).*

Length did not differ significantly between groups at any of the given timepoints. Condition factor was however significantly lower in the 5 spawning females in the LFD group 16th of March 2022 after approximately 1 year receiving different diet treatments (p=0.02) (Figure 10). For males, condition factor was significantly in the LFD group from 11th of August 2022 (p=0.03) (Figure 11).

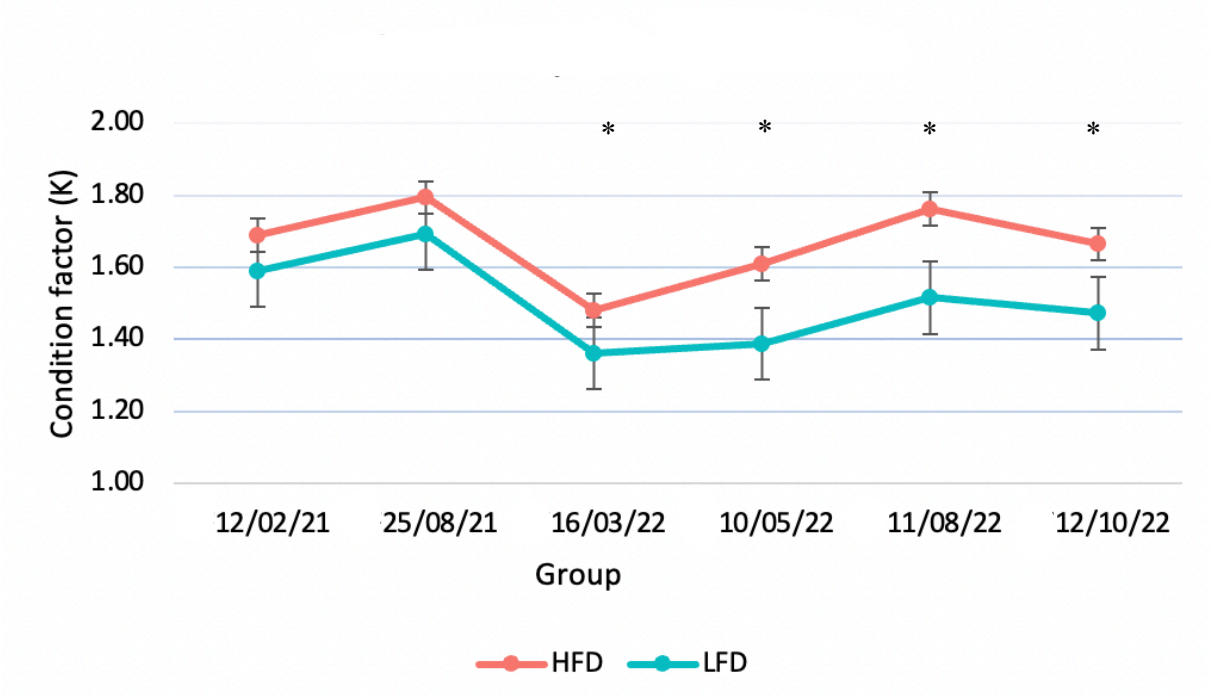

*Figure 10. Condition factor (K) for female spotted wolffish reared at different dietary treatments for two years (n=10, 5/group). Values are given as means ± SD. * Denotes significant differences between the two treatments (p<0.05).*

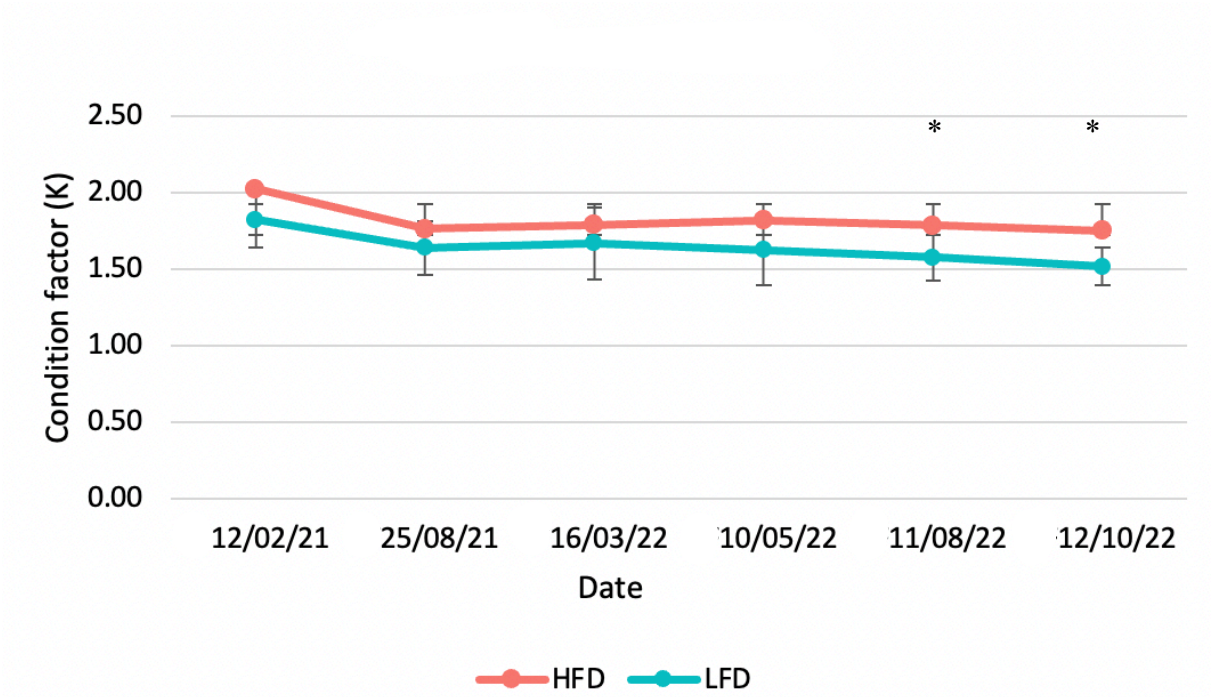

*Figure 11. Condition factor (K) for spotted wolffish males reared at different dietary treatments for two years (n=20, 10/ group). Values are given as means ± SD. *Denotes significant differences between treatments (p<0.05).*

Total fecundity was on average lower in the LFD group, but the difference was not significant. On average the LFD group spawned 7659 (±1315) eggs/female with volumes ranging from 1-1.4 l, whereas females in the HFD group spawned 10 264 (±1831) eggs/female with a volume of 1.6-2.0 l. There was no significant correlation between female weight after stripping and total fecundity. Egg diameter was on average 6.27 (± 0.14) mm in the LFD group compared to 6.06 (± 0.38) mm in the HFD group, but the difference was not significant. It was no significant correlation between egg diameter and female weight.

3.2 Gamete quality

3.2.1 Sperm quality

Considering the parameters evaluated using CASA, no significant differences were detected with respect to MOT, VCL or BCF (Figure 12). The LFD group (n=6) displayed slightly lower average values for all sperm motility parameters compared to the HFD group (n=8). MOT vas on average 47.6 (± 17.29) % in the LFD group compared to 65.7 ± (19.42) % HFD. VCL values was 23.8 (± 0.72) μms^{-1} in the LFD, and 24.64 (±1.91) μms^{-1} in the HFD group, whilst BCF averaged on 7.15 (±0.66) H_z in the LFD group compared to 7.97 (±1.67) H_z in the HFD diet group.

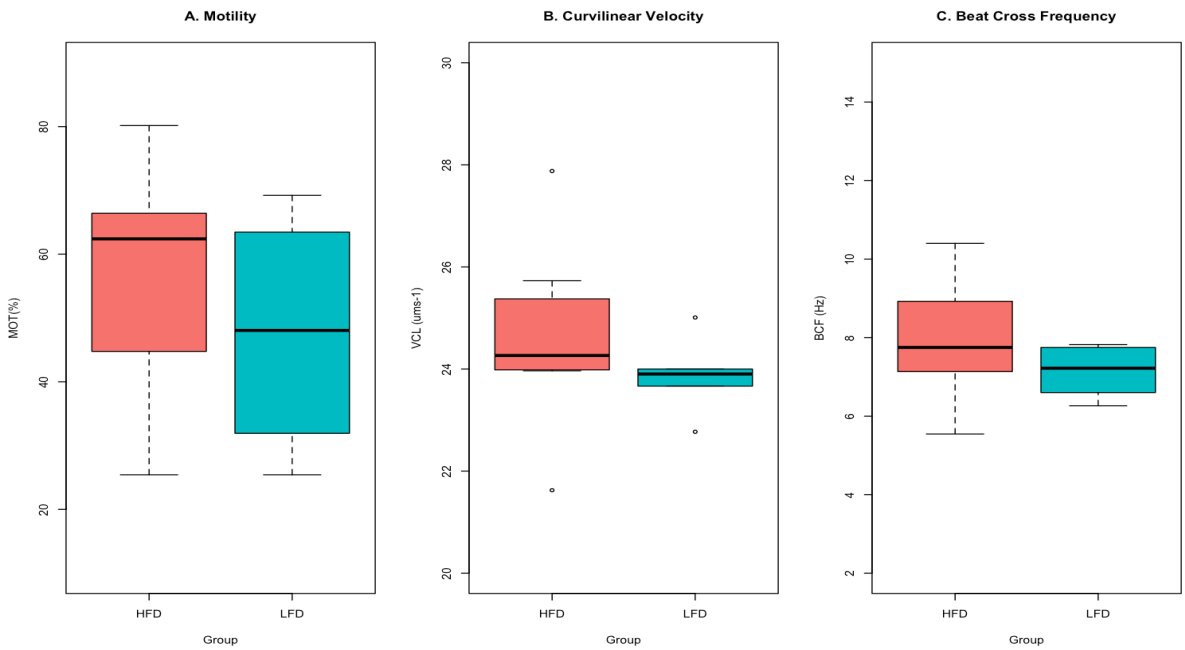

Figure 12. Sperm motility MOT (A), curvilinear velocity VCL (B), and beat cross frequency BCF (C) (results from CASA) in sperm samples for male spotted wolffish receiving different dietary treatments for two years. (LFD n=6, HFD, n=8). The box represents the interquartile

range, the black line inside the box is median, followed by stippled lines representing maximum and minimum values. Dots represents outliers.

Seminal fluid pH was on average 6.95 (± 0.49) in the LFD group, and 7.95 (± 0.23) in the HFD group (Figure 13). This difference was significant (p=0.03). No significant differences were detected for seminal fluid osmolarity between groups. Sperm samples from the LFD group displayed an average of 330.1 (± 7.4) mOsm/kg, compared to 332.2 (± 4.1) mOsm/kg in the HFD group. There was no significant correlation between pH and MOT. There was however a marginally positive correlation between pH and VCL (p=0.04, R=0.54) (Figure 14). Spermatocrit ranged from 1-7.1% in the LFD group and 1.3-7.4 % in the HFD group, and the difference was not significant. Sperm volumes did not differ significantly, but the LFD group produced on average 7.1 (± 4.5) ml compared to 5.8 (± 2.8) ml in the HFD group. There was no significant correlation between sperm volume and spermatocrit.

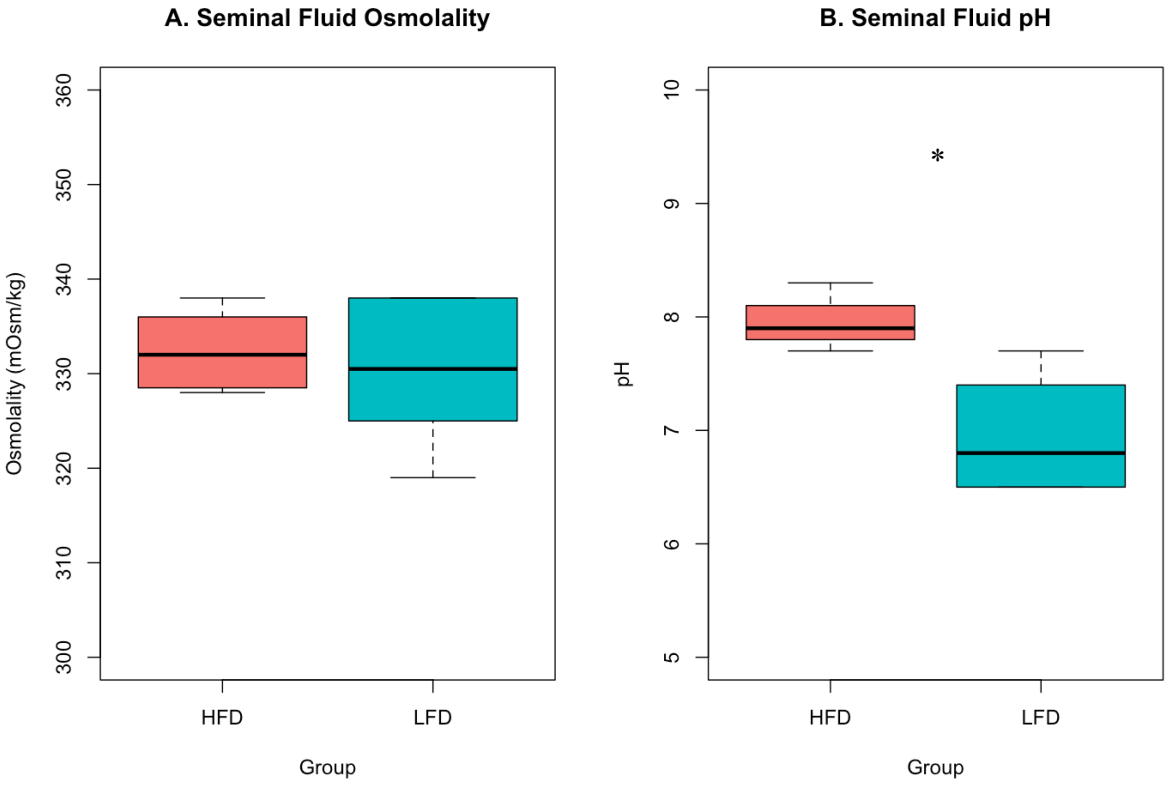

*Figure 13. Seminal fluid osmolality (A) and pH (B) in sperm from spotted wolffish males after receiving two different diets for two years (LFD n=6, HFD, n=8). The box represents the interquartile range, the black line inside the box is median, followed by stippled lines representing maximum and minimum values. * Denotes significant difference between the groups (p<0.05).*

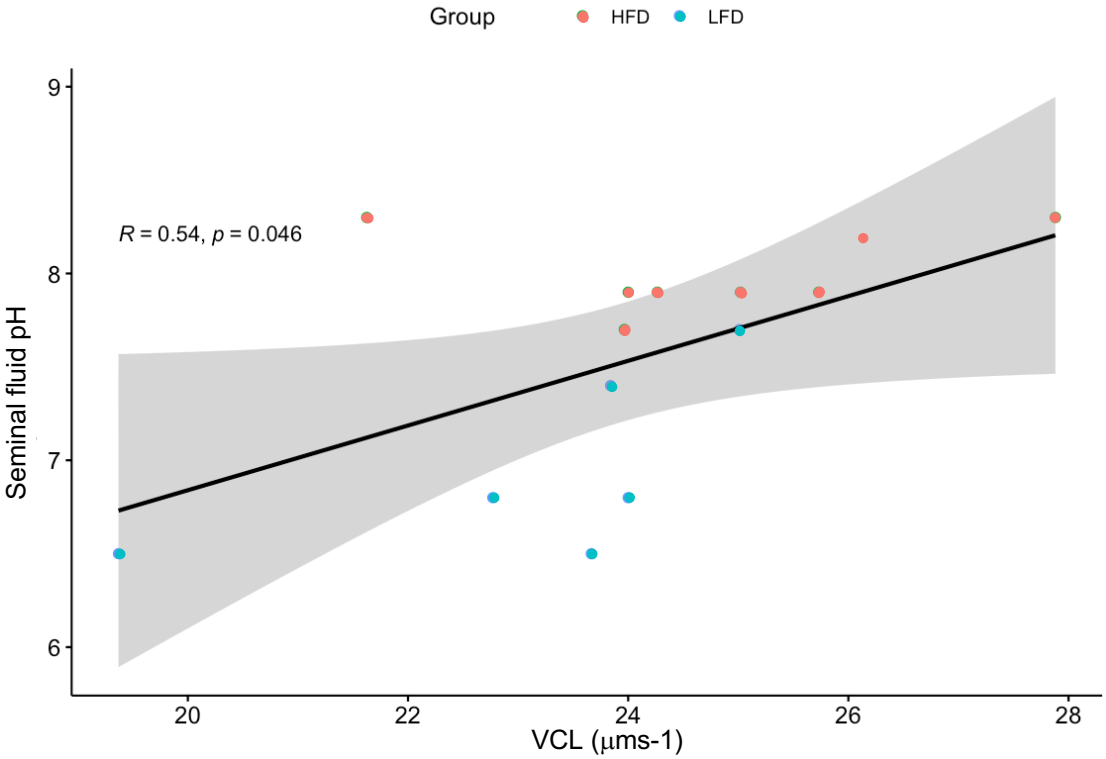

Figure 14. Correlation between curvilinear velocity (VCL) and seminal fluid pH of sperm samples from spotted wolffish males reared at two different diets for two years.

3.2.2 Egg quality

Fertilization rate (%) was high in both groups, on average higher in the LFD group, but the difference was non-significant. Evaluation of blastomere morphology at the 4-cell stage (n=8, 4/group) showed that 72% of eggs evaluated in the LFD group displayed normal morphology, compared to 55% of evaluated eggs in the HFD group. There was no correlation between blastomere morphology and survival throughout the incubation period, hatching rate, or deformity rate. An overview of fertilization rates and blastomere morphology are presented in Table 2.

Table 2. Overview of fertilization rate and proportion of abnormal, asymmetric, and normal cell cleavages in spotted wolffish eggs after broodstock received different dietary treatments (%) (n=8, 4/group). Values are given as means ± SD.

Group	Fertilization rate (%)	Abnormal (%)	Asymmetric (%)	Normal (%)
HFD	88 ± 13	18.2 ± 5.8	26.0 ± 10.7	55.6 ±16.5
LFD	96 ± 7	6.2 ± 9.5	17.5 ± 8.6	72.2 ± 18

Eggs from the LFD group displayed significantly higher survival to the eyed stage (p=0.02) and 1-week pre hatch (p=0.01) (See chapter 3.3). Survival varied between 52-96% in eggs from the LFD group, and 14-62% in eggs from the HFD group. Mortalities peaked between 50-300 DD. Content of fatty acids that have been identified as important for reproductive performance, in unfertilized eggs from the 5 spawning females from each group are presented in Figure 15. Fatty acid profiles of eggs from both groups were dominated by DHA, EPA, the SFA 16:0 and oleic acid. The LFD group displayed significantly higher concentrations of omega-3 fatty acids (p<0.001), EPA (p<0.001), AA (p<0.001), DHA (p<0.001) and polyunsaturated fatty acids (PUFA) (p=0.005) (Figure 12). The HFD group had significantly higher levels of omega-6 fatty acids (p=0.006) and omega-9 fatty acids (p<0.001), MUFAs (p=0.02), and significantly higher ratio between EPA:AA (p<0.001) (Figure 12). Full fatty acid profiles are given in Appendix 4.

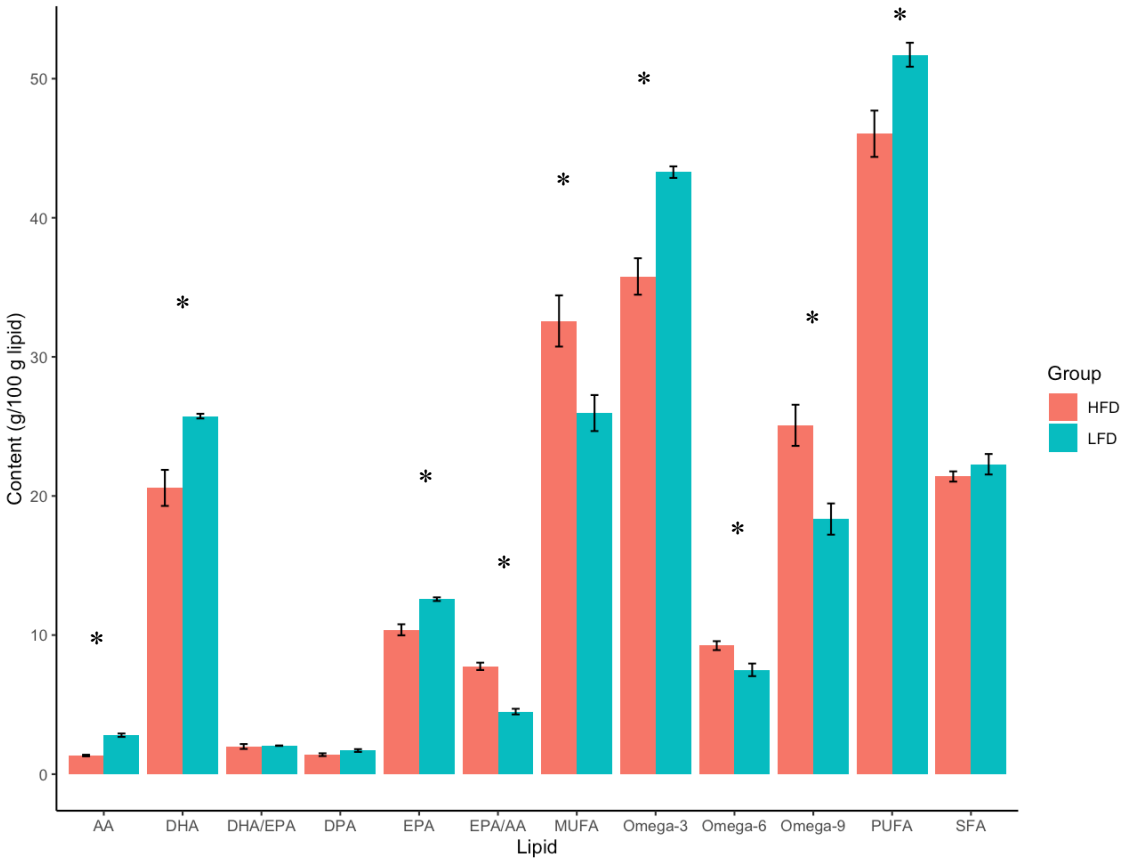

Figure 15.Concentration of lipids important for reproductive success in unfertilized eggs from spotted wolffish females reared at different dietary treatments for two years (n=10, 5/group). Values are given as means ± SD. Denotes significant difference between the groups (p<0.05).*

The concentration of SFAs, 22:5 n-3 docosapentaenoic acid (DPA), oleic acid, and ratio between DHA/EPA was not significantly different between the groups. A significant negative correlation between EPA:AA ratio (p=0.019, R=-0.72) and concentration of oleic acid (p=0.006, R= -0.79) on survival until 1 week pre-hatch was detected (Figure 16). There were significant positive correlations between EPA (p= 0.025, R=0.7), 16:0 (p=0.026, R=0.7), AA (p=0.025, R=0.7) and DHA (p=0.017, R=0.73), and survival to one week pre-hatch (Figure 17). There was also a significant positive correlation between n-3 fatty acids and survival to one week pre-hatch ($p=0.04$, R=0.6).

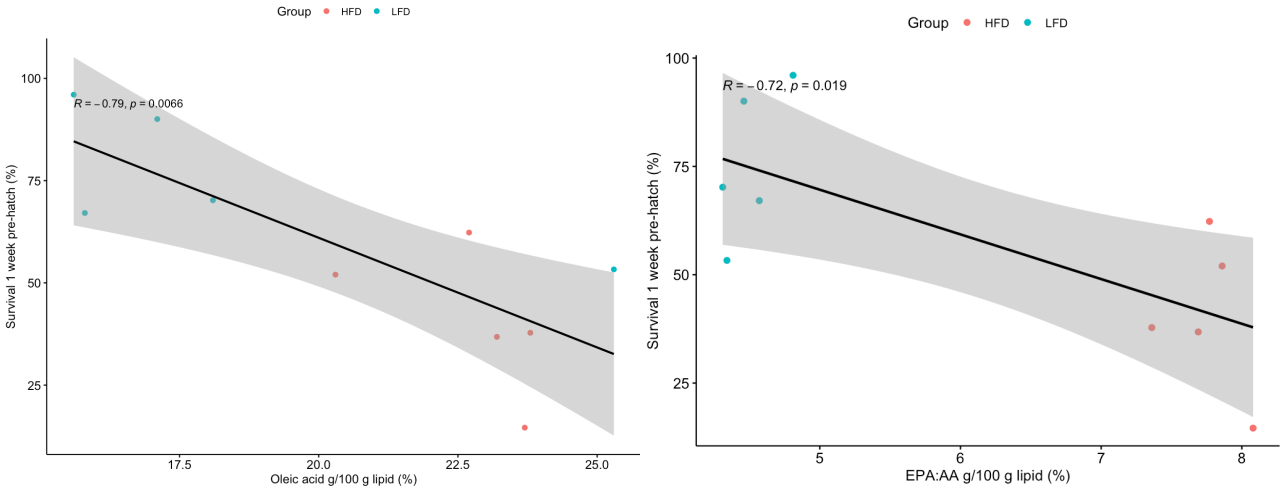

Figure 16. *Correlation plots for significant negative correlations between oleic acid (left) and EPA:AA ratio (right), and survival to one week pre-hatch (%) in eggs from female spotted wolffish receiving different dietary treatments for 2 years.*

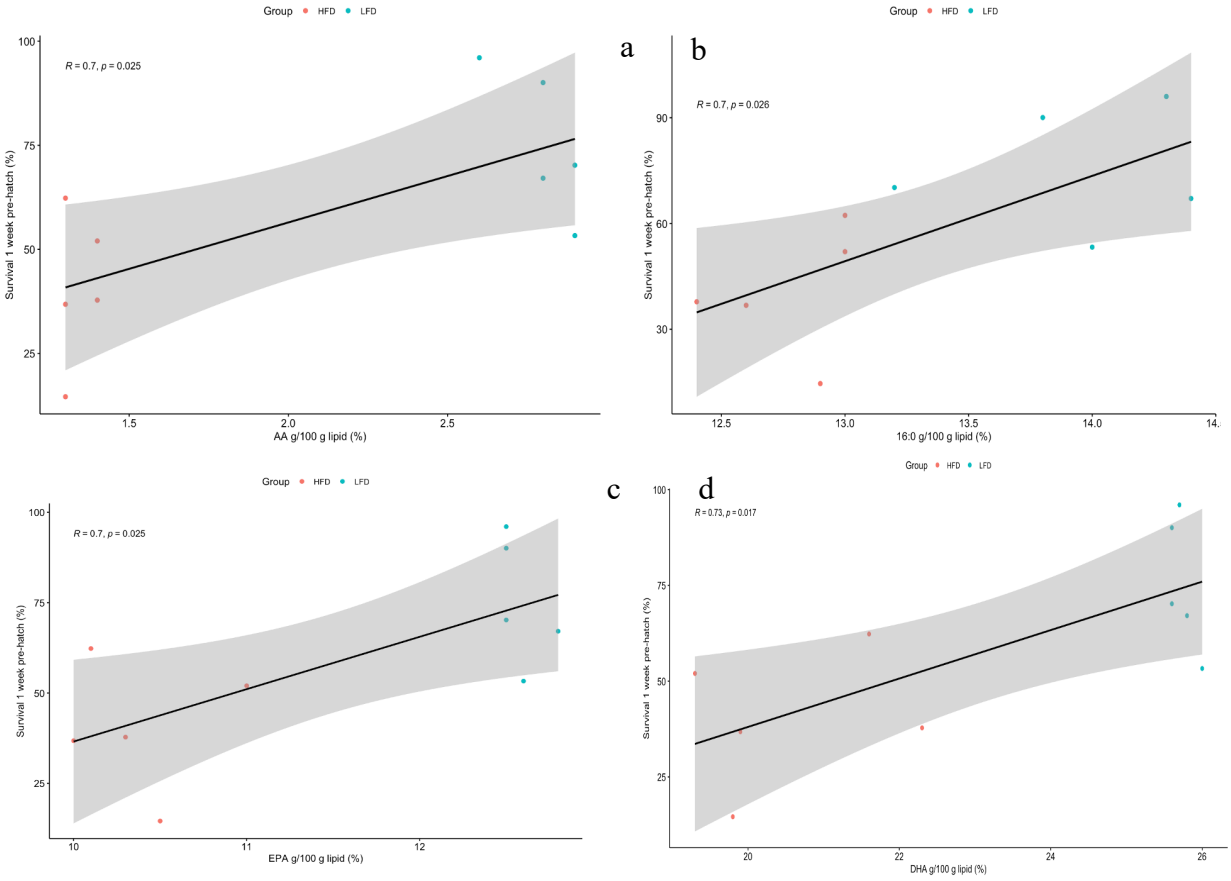

Figure 17. Significant positive correlations between concentration of AA (a), 16:0 (b), EPA (c), DHA (d) in unfertilized spotted wolffish eggs from females reared at different dietary

treatments for two years, and survival to one week pre-hatch.

3.3 Hatching rate and larvae quality

Hatching rate and survival to day 50 post hatch was on average higher in the LFD group compared to the HFD group (75% and 58% vs. 40% and 35% respectively), but the difference was not significant. Survival throughout the incubation time, hatching rate, and survival to day 50 post-hatch is presented in Figure 18. Failed hatching resulted in total mortality in of 2 out of 3 replicates from one egg batch from the LFD group. Mortality from day 0-50 post hatch varied from 4.7-49% in the LFD group and 10.5-36.4% in the HFD group. The difference was non-significant. The highest mortalities during the start feeding period were detected between day 30 and 40 post hatch. There were no correlations between fatty acids in unfertilized eggs and larval survival.

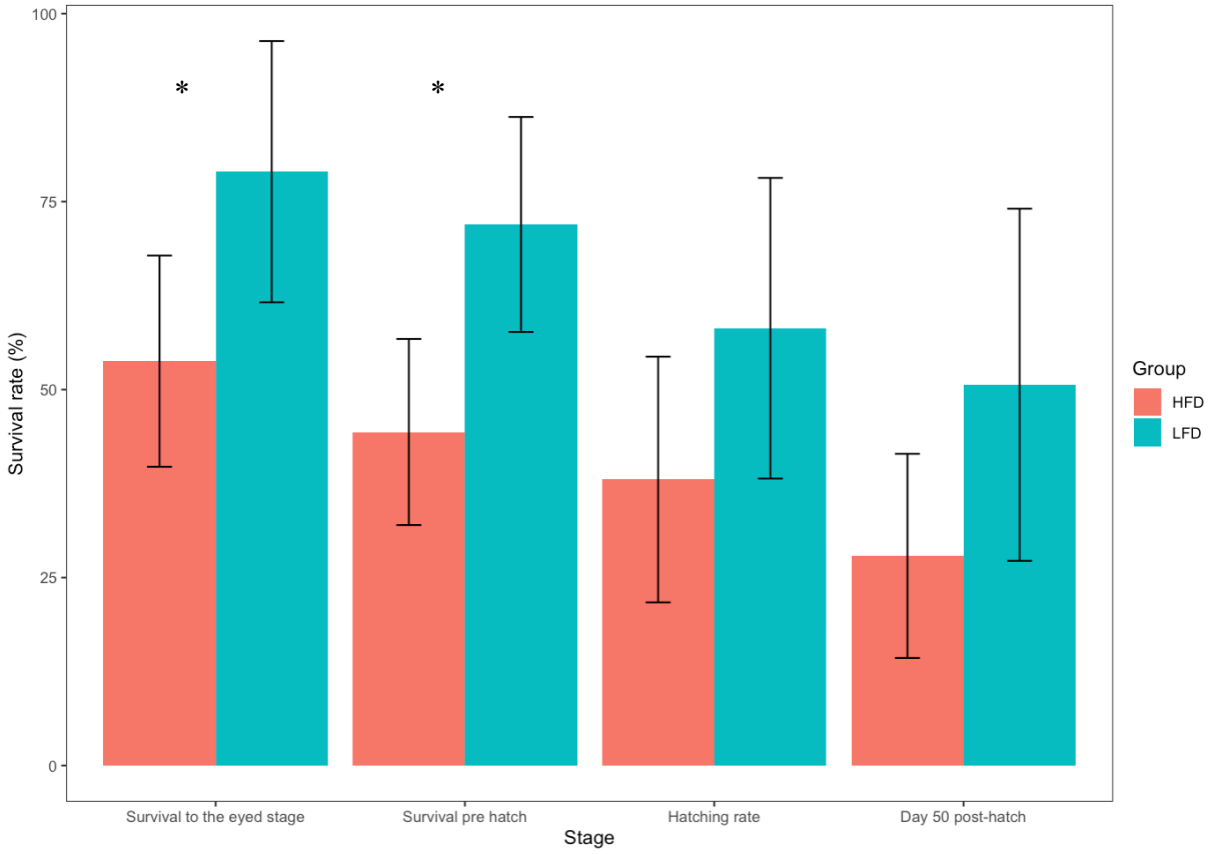

*Figure 18. Survival of wolffish eggs throughout the incubation period, hatching rate, and survival to day 50 post hatch in eggs from female spotted wolffish receiving two different diets. Values are given as means ±SD. * Denotes significant differences between treatments ($p<0.05$).*

Deformity rate was 6.2 (± 3.4) % in larvae from the LFD group compared to 9.2 (± 3.7) % in larvae from the HFD group. The difference was not significant. Larval length at day 0 did not differ significantly between the groups, with 22.2 (± 0.07) mm in the LFD group compared to 21.2 (± 0.34) mm in the HFD group. There was no significant correlation between egg size and larval length at day 0.

At day 50, larval length differed significantly between the two groups (p=0.02). The LFD group was on average 38.2 (± 1.6) mm, in contrast to 36.1 (± 0.9) mm in the HFD group. There was also a significant positive correlation between length at day 0 and 50 (p=0.006, R= 0.79), and between content of EPA and AA in unfertilized eggs and larvae length at day 0 (p=0.04, R=0.65, and p=0.01, R=0.74, respectively). Mean weight on day 50 did not significantly differ between the groups, as the average weight in the LFD group was 0.62 (±0.093) g in comparison to 0.59 (±0.096) g in the HFD group. Condition factor at day 50 post hatching was slightly lower in the LFD group compared to the HFD (1.1 (± 0.18) vs. 1.2 (± 0.13), respectively), but the difference was not significant. Larvae length at day 0, and length, weight, and condition factor on day 50 post-hatch are presented in Table 3. An overview of all correlations tested in this study is presented in Appendix 5.

Table 3. Larval length at day 0, and length, mean weight, and condition factor day 50 post hatch in larvae from broodstock receiving different dietary treatments. Significant differences are highlighted in bold (p<0.05). Values are given as means ±SD.

Parameter	HFD	LFD
Larvae length day 0 (mm)	21.22 ± 0.3	22.23 ± 0.07
Larvae length day 50 (mm)	36.18 ± 0.9	**38.30 ± 1.5**
Larvae weight day 50 (g)	0.59 ± 0.09	0.62 ± 0.09
CF day 50 (K)	1.26 ± 0.13	1.11 ±0.18

4 Discussion

Variability in gamete quality and subsequently high mortalities during the earliest life stages has been identified as one of the main bottlenecks to solve to upscale commercial production of the spotted wolffish (Beirão & Ottesen, 2018). Broodstock nutrition is recognized as crucial for gamete quality and reproductive success in fish in general (Brooks et al., 1997), yet no species-specific formulated diet has so far been developed for the spotted wolffish. In the present study, an experimental low-fat broodstock diet, closely resembling the nutritional content of the natural diet of the spotted wolffish, was designed to investigate the impact of diet on egg, sperm, and larval quality. The results demonstrated that the low-fat broodstock diet resulted in higher egg-survival throughout the incubation period, and bigger larvae at day 50 post hatch, but a lower mean weight and condition factor in the parental fish receiving this diet. Diet did not appear to influence sperm quality remarkably under the present experimental conditions. The impact of broodstock diet in wolffish will be discussed in terms of fish growth and fecundity, gamete and larval quality and compared with findings reported in the same species and a range of other cultured species. This is in the knowledge that these traits depend on a vast array of factors and co-factors which may vary greatly at individual and stock level in close and distantly related species displaying varied dietary requirements.

4.1 Broodstock growth and fecundity

Total fecundity is a measure of the number of eggs produced over a spawning season (Izquierdo et al., 2001). The spotted wolffish are determinate spawners, and all oocytes are matured and ovulated during one spawning episode each season (Pavlov & Moksness, 1996). Results from this study showed that the two diet groups did not differ in terms of total fecundity. There was however a trend for the 5 spawning females from the LFD group produced a lower number and volume of eggs on average. In addition, the mean weight and K-factor was significantly lower for the 5 spawning females in the LFD group one year into the pre-experimental feeding period. This also applied for all males, where mean weight and K-factor was significantly lower in the LFD than in the HFD group approximately one year into the pre-experimental feeding period. Length did not differ significantly between groups at any of the given timepoints.

Fecundity has been correlated to body size of the spawning female (Rennie et al., 2005). Strong correlations have been found between female wolffish weight and eggs spawned during a spawning season, where larger females produced higher number of eggs (Falk-Petersen et al., 2001; Pavlov & Moksness, 1996). This is in contrast to findings from this study, where a weak,

but not significant, correlation was found between fecundity and female weight. There was however a tendency for females in the LFD group to produce lower volumes of eggs, where also K-factor and mean weight was significantly lower than in the HFD group. Krill meal as a protein source in fish diets have previously reported to improve growth performance (Torrecillas et al., 2021), but this was not reflected in results from the current study.

Broodstock diets have shown to impact fecundity (Da Silva et al., 2016; El-Sayed et al., 2005; Zakeri et al., 2011). Zakeri et al., (2011) found that lipid content of broodstock diet affected fecundity in the yellowfin sea bream (*Acanthopagurs latus*), where an increase in lipid content showed a positive impact on fecundity. This has also been reported for other species like channel catfish (*Ictalurus punctatus*), where Sink & Lochmann (2008) found that a 10% increase in lipid content had positive effects on spawning success in terms of fecundity. In the experimental LFD diet in the current study, lipid content was reduced by almost 40%, which may explain the decrease in weight and condition factor in the LFD group. Jonassen (2002) however found indications that the spotted wolffish has a limited capacity of utilizing fat as an energy source, as fish fed a diet with a fat content of 15% displayed higher growth rates compared to fish fed a diet with 20% fat. The findings from the present study, where growth was lower in the low-fat diet group, is in contrast to previous results, and could indicate that the fat content in the LFD-group might have been too low to enhance growth.

Fecundity may also be linked to the source of protein in broodstock diets. Vassallo-Agius et al., (2001) found that fecundity decreased for striped jack (*Pseudocaranx dentex*) females fed a diet where 50% of the fishmeal was replaced with krill and squid meal. Other studies have reported the quite opposite, that squid meal increased fecundity for the gilthead sea bream, *Sparus aurata* (Harel et al., 1994). In the LFD, squid meal was the main protein source accounting for 30% of the total diet content, and there were no indications that squid meal increased fecundity. Squid meal is however generally considered to positively affect egg quality in several fish species, due to its beneficial lipid and protein content (Emata et al., 2003; Fernàndez-Palacois et al., 1997; Vassallo-Agius et al., 2001).

Egg size (mm) was not significantly different between groups but there was a tendency for females in the LFD group to produce bigger eggs. It has been indicated that eggs of larger size contain larger yolk volumes, which is presumed to be beneficial for the larvae (Baynes & Howell, 1996; Brooks et al., 1997). Stuart et al., (2020) found positive correlations between egg diameter and viability for the California yellowtail (*Seriola dorsalis*). Egg size has also

been correlated with larval size, where bigger eggs produced bigger larvae at hatch (Gisbert et al., 2000; Springate & Bromage, 1985). Increased content of vitamin A and E have previously been correlated to initial egg size for the turbot (*Scophthalmus maximus*) (Lavens et al., 1999). In the present study, there was no correlation between egg size and larvae size at day 0. However, the experimental LFD group produced bigger eggs and larvae on average, indicating that there may be a dietary impact on egg size and following larvae size.

There was no correlation between female weight and egg size, which aligns with findings from other studies on the spotted and common wolffish (Falk-Petersen et al., 2001; Pavlov & Moksness, 1996). Egg size may, however, be affected by other environmental and physiological factors such as number of spawnings, photoperiod and age of the female (Campos-Mendoza et al., 2004; Dupont-Cyr et al., 2018). In the present study, the spawning females from both diet groups were of the same age, and subjected to the same photoperiod. There have been indications that first time spawners may produce smaller and less viable eggs compared to second time spawners (Dupont-Cyr et al., 2018; Hansen & Falk-Petersen, 2002b), but this could not be verified in this experiment.

4.2 Gamete quality

4.2.1 Sperm quality

The only significant difference between the two groups in terms of sperm quality was seminal fluid pH. Generally, seminal fluid pH has been correlated with motility, where sperm samples with pH values higher or lower than the optimum displayed lower motilities (Effer et al., 2013; Wojtczak et al., 2007). In this study, no correlation between pH and sperm motility (MOT) was found in spite of significant differences in seminal fluid pH recorded between the experimental and commercial diet groups, averaging 6.9 and 7.9, respectively. Kime & Tveiten (2002) found that pH had little impact on sperm motility characteristics for the spotted wolffish, where only a pH value as low as 4.5 significantly decreased MOT, VCL and BCF under their experimental conditions. In the present experiment, there was no significant correlation between seminal fluid pH and MOT. There was however a significant positive correlation between seminal fluid pH and other sperm parameters like VCL, indicating that seminal fluid pH may have affected sperm motility characteristics. pH values in the present experiment were however slightly higher compared to Kime & Tveiten (2002).

In the present study, the seminal fluid osmolality was similar in both diet groups ranging from 310-340 mOsm/kg and in line with other studies on this species (Gonzàles-Lopez et al., 2021; Kime & Tveiten, 2002). Kime & Tveiten (2002) found that osmolality values between 200-400 mOsm/kg appeared to be the optimum for spotted wolffish sperm. For other marine fish species, osmolality values have been found to range between 290-514 mOsm/kg (Zadmajid et al., 2019). Urine contamination may also be linked to seminal fluid pH and osmolality in sperm samples. For example, Gonzáles-Lopez et al., (2021) found that spotted wolffish sperm retrieved through stripping where urine was initially expelled prior to sperm collection had still a slightly, but not significantly higher concentration of urine compared to sperm obtained directly from the testis. This indicates that the performed procedure for sperm collection in this study should be appropriate to avoid unnecessary contamination of urine.

Specific amino acids have been reported to impact sperm quality (Butts et al., 2020). Amino acids are crucial compounds in sperm cells and seminal fluid and play a key role in protecting the sperm cells during spermatogenesis (Kwasek et al., 2014; Mansour et al., 2006). In particular, alanine, arginine, and glutamic acid have all been associated with higher reproductive success in terms of sperm quality in common barbel, *Barbus barbus* (Alavi et al., 2009). Butts et al., (2020) found that increased dietary arginine increased seminal fluid pH in the European eel (*Anguilla anguilla*), and that specific concentrations of amino acids in broodstock diet altered sperm quality, where males fed enriched diets displayed higher sperm MOT and VCL. In the present study, the amino acid composition of the HFD is not known, therefore no conclusion regarding the possible impact of dietary amino acids on sperm quality can be drawn in this experiment.

Generally, results from CASA parameters showed a high degree of individual variance within the two diet groups. VCL was lower in this experiment compared to Kime & Tveiten (2002). In the present study values averaged at 23.8 μms^{-1} for both groups, compared to 50 μms^{-1} in their study. Other studies on the other hand refer to VCL of 20.6 μms^{-1} for spotted wolffish sperm (Gonzáles-Lopez et al., 2021). BCF was however similar compared to Kime & Tveiten (2002). For MOT, high variance was also seen between replicates from the same fish (e.g., 46% vs. 86% and 39% vs. 65%). This deviation was seen for 4 out of 6 fishes in the LFD group, highly affecting average values in this group. The lowest value was always recorded in the first replicate. According to expectations, the second replicate should have had lower MOT due to slightly longer storing time in the fridge while recording the first replicate. However, CASA is

generally considered as a powerful tool for precise objective estimates of sperm motility characteristics (Cabrita et al., 2014; Gallego et al., 2018).

The spotted wolffish produces lower amounts of less dense sperm compared to other species. The low sperm volumes are most likely due to the nature of their reproductive strategy of internal fertilization, where only small volumes are needed (Beirão et al., 2020; Kime & Tveiten, 2002; Le Francois et al., 2007). Spermatocrit of the spotted wolffish is variable and commonly ranging 1-17% (Beirão & Ottesen, 2018; Le Francois et al., 2021a), which is also reflected in findings from this study with values varying from 1 to 7% in both treatment groups. This is in contrast to other species like Atlantic cod and the polar cod (*Boreogadus saida*) commonly displaying spermatocrit values >90% (Bender et al., 2016). In the present work, sperm volumes at stripping ranged from 1-15 ml, and there was no significant correlation between sperm volume and spermatocrit. As such, there were no significant differences between the groups in terms of spermatocrit or sperm volumes, and no strong evidence that diet affected sperm density.

Fatty acid composition of broodstock diets have been correlated to sperm quality (Baeza et al., 2015; Beirão et al., 2015; Mansour et al., 2011). Generally, broodstock diets enriched with PUFAs have been shown to enhance reproductive performance in terms of sperm quality (Asturiano et al., 2001; Butts et al., 2015). Nyina-Wamwiza et al., (2012) found that the replacement of fish meal with PUFA rich compounds improved sperm motility in the African catfish (*Clarias gariepinus*). Baeza et al., (2015) suggested that EPA levels in the liver contributed to the produced sperm volume in the European eel. In their study, AA, and EPA:AA was positively correlated to sperm VCL and density respectively. The slightly increased sperm volumes in the LFD group in the present study may be correlated to the higher levels of dietary EPA, but this is not verified as sperm lipid analysis was not performed in this trial. Beirão et al., (2015) found that sperm quality in the Senegalese sole (*Solea senegalensis*) significantly increased in broodstock fed a diet rich in DHA with additional antioxidants and vitamin E. Both vitamin C and E are thought to play a protective role for the sperm cells, thus improving quality and fertilization ability (Alavi et al., 2005; El-Gamal et al., 2007). Despite the higher concentrations of both vitamin C and vitamin E in the LFD compared to the HFD, sperm quality was not significantly improved in this group. Nevertheless, this did not affect the sperms' ability to fertilize the eggs, as fertilization rates were high in both groups.

4.2.2 Egg quality

Survival until certain stages during the embryogenesis have been considered as a powerful determinant for assessing egg quality in fish, especially for species requiring longer incubation time (Migaud et al., 2013). In the present study, survival to the eyed stage (400 DD) and 1 week pre-hatch was significantly higher in the LFD than in the HFD group. There were significant positive correlations between survival throughout the incubation time and content of EPA, n-3 fatty acids, 16:0, DHA, and AA, suggesting that the increased survival could be connected to the lipid composition of the eggs. In this study, concentrations of fatty acids differed significantly between the two groups, indicating that broodstock diet did impact lipid composition of unfertilized eggs. This is in agreement with Morais et al., (2014), who found clear indications that dietary essential fatty acids were incorporated in the oocytes.

Tveiten et al., (2004) investigated the fatty acid profiles of unfertilized spotted wolffish eggs and the utilization of these during the embryonic development. Their findings suggest that DHA, AA and 16:0 were conserved during embryogenesis, and that EPA may function as an energy source during embryonic development. Generally, EPA has been positively correlated to egg survival for other fish species (Curzado et al., 2010; Xu et al., 2019). The ratio between EPA:AA has also been pointed out as highly important for larval development and survival in marine fish, where increased ratios have been presumed to be beneficial for some fish species (Bell et al., 1997; Mazorra et al., 2003). This is in contrast to findings from this study, where a significant negative correlation between EPA:AA and survival to one week pre-hatch was observed. Similar results were reported by Tveiten et al., (2004), who also characterized eggs from the spotted wolffish with lower ratios of EPA:AA compared to other teleost fish species.

Generally, lipids obtained from the yolk and oil droplets are considered as important for energy production during the embryogenesis in the spotted wolffish (Desrosiers et al., 2008). In the present study, the highest mortality rates for eggs were recorded between 50 and 300 DD in both groups. This was also described in previous studies for the spotted wolffish (Falk-Petersen et al., 2001; Falk-Petersen & Hansen, 2003). The organogenesis occurs from approximately 90 DD (Falk-Petersen & Hansen, 2003), suggesting that observed mortalities during this period was linked to failed development of vital organs. Eggs from the LFD group had significantly higher concentrations of EPA, AA and DHA, and significantly lower AA:EPA ratio compared to eggs from the HFD group, which appeared to be beneficial for egg quality in the present study. There have however been reports of DHA influencing egg quality negatively for the brill,

Scophthalmus rhombus (Curzado et al., 2010). In addition, Wing-Keong & Wang (2011) found no evidence that increased dietary DHA or EPA affected egg quality in Nile tilapia.

Eggs of good quality also contained high levels of n-3 fatty acids in this experiment, agreeing with findings for other species (Fernàndez-Palacois et al., 1995; Zakeri et al., 2011). Harel et al., (1994) concluded that n-3 fatty acids were responsible for improvement of egg quality in gilthead seabream, in agreement with Callan et al., (2012) where an increase of 1.3% dietary n-3 HUFAS significantly increased spawning success and egg quality for the angel catfish (*Centropyge loriculus*). This is however in contrast to findings for the Japanese flounder (*Paralichthys olivaceus*), where concentration of n-3 fatty acids was negatively correlated to egg survival (Furuita et al., 2003). The balance between n-3 and n-6 fatty acids has been pointed out as more important for egg survival than the concentration of exclusively n-3 fatty acids (Bell & Sargent, 2003).

In this experiment, the fatty acid profiles of unfertilized eggs were dominated by oleic acid, EPA, DHA and the SFA 16:0 in both diet groups. This aligns with the findings of Tveiten et al., (2004) on the spotted wolffish, and results from Ringøe et al., (1987) for the common wolffish. EPA and DHA are also reported to be the dominating fatty acids in eggs from other cold-water species like the Atlantic salmon (*Salmo salar*) and Atlantic halibut (*Hippoglossus hippoglossus*) (Kowalska-Gòralska et al., 2020; Mommens et al., 2015; Silversand & Haux, 1995). Oleic acid has previously been reported to negatively impact egg quality in captive lumpfish, *Cyclopterus lumpus* (Pountney et al., 2022). In the present study, there were significant negative correlations between concentrations of oleic acid and survival to hatching, even though it was one of the most abundant fatty acids measured in both treatment groups. Oleic acid has been considered as an important source of energy during the egg-phase in the greater amberjack (*Seriola dumerili*) (Sarih et al., 2020). Similar indications have also been reported for the spotted wolffish (Tveiten et al., 2004). In this study, oleic acid in unfertilized eggs appeared to be beneficial at given concentrations.

Vitamins obtained from broodstock diets are vital for several metabolic processes (Sarmento et al., 2018). In particular, vitamin A, C, D and E have been identified as critical essential compounds for reproductive performance in fish (El-Gamal et al., 2007; Hamre et al., 2010). Teleost fish are unable to synthesize certain vitamins, and most are essential, derived from the diet (Darias et al., 2011). Vitamin E functions as an antioxidant and plays a vital role in protecting the egg during their initial stages of development, whilst vitamin A is required for

embryonic development in fish (Palace & Werner, 2006). Both vitamin C and D have been correlated with development of the skeletal system (Darias et al., 2011). Furuita et al., (2009) found that injection of vitamin C and E in the Japanese eel improved egg and larvae quality, which agrees with findings from other studies on other species (Eskelinen, 1989; Sarmento et al., 2018). Vitamin C deficiencies have previously been reported to decrease egg quality (Watanabe et al., 1991). From the two diets tested in this trial, LFD contained 271 mg/kg more vitamin C compared to HFD, which may also explain the increased survival of eggs throughout the incubation period.

Brooks et al., (1997) discussed how broodstock fed diets resembling their natural diet often induce an increase in reproductive success. A general issue in aquaculture is linked to failed development as a result of proper nutritional requirements not being met. The strongest positive correlations in terms of egg survival were between AA and EPA in this study. In the wild, wolffishes feed on prey rich in n-3 HUFAs, especially EPA and DHA, in addition to AA (Silversand & Haux, 1995). Accordingly, this may explain why high concentrations of AA and EPA in unfertilized eggs appeared to be beneficial for egg survival in this experiment too. The natural prey of wolffish is also classified with low lipid contents (Le Francois et al., 2021a). Both AA and EPA are thought to be important lipids for production of eicosanoids, a compound associated with increased susceptibility to environmental stress during the embryogenesis (Wilson, 2009). Findings from this study agree with conclusions from Sargent et al., (1999), who suggested that all DHA, EPA, and AA are important for early development in marine fish, but that optimum concentrations and ratios will be species specific.

Disinfection of egg surfaces during the incubation period of the spotted wolffish is necessary to avoid bacterial destruction of the eggshell and early hatching (Ellingsen et al., 2005; Hansen & Falk-Petersen, 2001; Pavlov & Moksness, 1993). In the present work, results for hatching rates were highly affected by failed hatching in 2 out of 3 replicates in one egg batch from the LFD group. The failed hatching was likely linked to improper disinfection procedures. This was the first batch being incubated for this experiment, and a higher initial volume of eggs were used (0.15 l per replicate), which was then reduced for subsequent egg batches. All batches initially received the same treatment throughout the incubation period. Due to the higher egg amount, and thus thicker egg layer, it is likely that the disinfectant was trapped between the egg layers, indirectly treating the eggs for longer than recommended.

Hansen & Falk-Petersen (2001) found that disinfection with too high concentration of the sanitizer throughout the incubation period resulted in a hardening of the eggshell, and inhibition of hatching resulting in total mortality. Similar observations were registered in the present experiment. The batch where hatching failed displayed 96% survival throughout the incubation period in all three replicates, whereas hatching rate was 91% in the non-affected replicate. Hypothetically, if all three replicates displayed equal hatching rate as the non-affected replicate, the difference between the groups in terms of hatching rate would be significantly higher for the LFD group (p=0.03). With the failed hatching, the hatching rate for the whole egg batch is reduced to 30.5%, making the difference non-significant. Considering the higher number of eggs compared to the rest of the batches, this batch should have been discharged and replaced.

Abnormal blastomere cleavage has been associated with egg mortality, low hatching rates and larval abnormalities (Hansen & Falk-Petersen, 2002a; Kjørsvik, 1994; Pavlov & Moksness, 1996; Shields et al., 1997; Tveiten et al., 2001). In this study, no correlations were found between blastomere morphology at the 4-cell stage and egg quality determinants. This is in contrast to other studies on the wolffish (Hansen & Falk-Petersen, 2001; Tveiten et al., 2001). In the present study, there was a tendency that eggs from the LFD displayed higher incidence of normally developing eggs. Avery et al., (2009) suggested that eggs with early abnormal cleaving may develop normally later in the incubation period, and proposed a grading of the severity grade where less severe abnormalities may have little impact on the embryonic development. In the present study, blastomere cleavage was not performed on the first batch from each group due to issues with the fixation. These two batches displayed the highest and lowest survival throughout the incubation period (96% and 14% respectively), and correlations may have been present if these were evaluated.

For species like the salmonids, fertilization rate has also been acknowledged as a good predictive indicator for egg viability (Shields et al., 1997). In the present work, egg fertilization rate was high in both groups (>88%). Beirão & Ottesen (2018) discussed the optimum egg to sperm ratio after sperm density for the spotted wolffish, but this was not considered in the present study. Contact time between eggs and sperm was standardized to 3 hours, and a fixed amount of 3 ml sperm per 1.5 dl egg was used. Generally, 2 ml of high-quality wolffish sperm is sufficient to fertilize 1 l of eggs in aquaculture practice (Beirão & Ottesen, 2018; Santana et al., 2020). A lack of the optimization of the contact time after sperm quality determinants might

have impacted the fertilization rate. However, considering the high sperm amount for the low amounts of eggs, 3 hours contact time should be enough to obtain sufficient fertilization.

4.3 Larval quality

Initial egg quality plays a crucial role determining the overall survival (Hansen & Falk-Petersen, 2002a; Kjørsvik et al., 2003). Larval quality was determined through deformity rate, survival, and growth until day 50 post hatching. Larvae from the LFD group displayed lower mortality rates compared to the other group from day 0 to 50 post hatch, but the difference was not significant. There was also a tendency for larval length on day 0 to be higher in this latter group, in addition to lower deformity rates, but the difference was, again, non-significant. Larval length on day 50 was however significantly higher in the LFD vs. the HFD group, but mean weight and K-factor did not vary between the two diet groups.

Some level of skeletal malformations were observed in the present study. Similar observations have been reported by Hansen & Falk-Petersen (2002b) for the spotted wolffish, where the deformity incidence was higher in eggs incubated at higher temperatures. Deformity rates have been correlated to fatty acid composition of broodstock diets in species like the Nile tilapia (Wing-Keong & Wang, 2011). In the present study, there was no correlations between fatty acid composition of unfertilized eggs and deformity rates. Vitamin content of broodstock diets have also been associated with incidence of larval skeletal deformities in other fish species, where too high levels of vitamin A have been associated with increased deformity rates (Cahu et al., 2003; Lewis-McCrea & Lall, 2010). Similar observations have been reported for vitamin C deficiencies for some fish species (Fraser & de Nys, 2011; Madsen & Dalsgaard, 1999).

Even though larvae of spotted wolffish hatch at an advanced stage of development, mortalities frequently occur during the start feeding period (Hansen & Falk-Petersen, 2002a). Day 0-50 post hatching is acknowledged as the most critical period for the wolffish larvae, due to absorption of the yolk sac and transition to exogenous feeding (Foss et al., 2004; Hansen & Falk-Petersen, 2002b; Strand et al., 1995). Failure to initiate feeding has previously been reported as the major cause of mortalities during this period for the common wolffish, where Strand et al., (1995) reported that death caused by starvation occurred 30-40 days post hatch. This is also when mortality rates peaked in the present study. Accordingly, it is likely that observed mortalities during this period was related to failed initial feeding. Similar findings

were also discussed in other studies of spotted wolffish larvae (Falk-Petersen et al., 2001; Savoie et al., 2006).

Larval performance and survival have been correlated to lipid composition of the yolk sac in some fish species (Henrotte et al., 2010; Mazorra et al., 2003; Wilson, 2009). In this work, there was no correlation between fatty acid composition of unfertilized eggs and larval survival. There was however a significant positive correlation between length at day 0 and day 50 in the present study, indicating that the length differences persisted throughout the start feeding period. Similar results have also been reported by Falk-Petersen et al., (2001) and differences in larval length were assumed to be correlated to larger initial egg size (Gisbert et al., 2000). In this study, there were no correlations between egg diameter and larvae size at day 0, but there was a positive significant interaction between content of AA and EPA in unfertilized eggs and larval size at day 0.

Even though larvae from the LFD group on average displayed lower mortality rates during the start feeding period, the difference was non-significant between the two groups. This is linked to 49% mortality in one batch from the LFD group, compared to values varying from 4.7-12.5% in the remaining 4 batches. Again, this was the batch that displayed the highest survival to one week pre-hatch (96%). The high mortality seen in this specific batch could be due to environmental factors, such as density of fish and food availability, and as such the number of larvae should have been modified and better standardized to avoid such deviations.

Larval performance may also be a result of genetic factors and environmental conditions. Parental genes have shown to greatly influence performance during the earliest life stages (Labbé et al., 2017; Morais et al., 2014). Density and number of fish in each experimental unit during the start-feeding period was not standardized, as the initial number of hatched larvae which varied between batches. This could have influenced food availability, even though the amount of feed was adjusted according to biomass present. However, Hansen & Falk-Petersen (2002b) found indications that spotted wolffish may grow faster at higher densities, as larvae reared in densities of 3110 m^3 displayed higher growth rates compared to larvae reared at a density of 1555 m^3. Protocols for weaning of the spotted wolffish are yet to be optimized, and considerable R&D work in this direction still remains. Environmental parameters such as water temperature and quality have also been correlated to larval performance of the wolffish (Foss et al., 2003; Moksness et al., 1989), but since all start feeding units received water from the same source it is unlikely that this influenced the results remarkably. In this sense, the shallow

raceways used in this work is considered to be well suited for start feeding of wolffish (Foss et al., 2003; Strand et al., 1995).

5 Conclusion

In conclusion, diet manipulation of a spotted wolffish broodstock did impact reproductive performance under the present experimental conditions. Broodstock weight and condition factor were significantly reduced in the experimental LFD group. There was no difference in terms of fecundity or egg size. It was however a tendency that females in the LFD group spawned lower amounts of bigger eggs. Fertilization rates and blastomere morphology at the 4-cell stage did not vary between groups. Sperm quality determinants was not remarkably affected by different dietary treatments, and the only significant difference detected was lower seminal fluid pH in sperm samples from the LFD group, which marginally affected sperm motility characteristics in terms of VCL. The sperms' ability to fertilize the eggs was however high in both groups.

Dietary treatment significantly improved embryo survival from incubation to 1-week pre-hatch, which was higher in eggs from the LFD group. Embryo survival was positively correlated to lipid composition of eggs (EPA, DHA, AA, n-3 fatty acids and 16:0), which differed significantly between groups, indicating that different dietary treatments altered the chemical composition of eggs. Negative correlations between embryo survival and concentrations of EPA:AA ratio and oleic acid was detected. Generally, it appeared that eggs from the LFD group contained beneficial levels of essential fatty acids. Hatching rates did not vary between groups, likely due to the failed hatching of one high-quality egg batch from the LFD group.

Larval survival, size and growth was also seemingly higher in the LFD group, albeit not significantly. Larval length on day 50 was however significantly higher in larvae from the LFD treatment. Survival from incubation to day 50 post hatch increased from 35% (HFD group) to 58% (LFD group) after broodstock received different dietary treatments, which marks a potential for great improvement in the commercial production of spotted wolffish. Further studies are still needed to validate the present results and to further optimize diet for spotted wolffish broodstock.

6 Future perspectives

The present study found that diet did impact egg quality in terms of survival throughout the incubation time, although the exact mechanisms behind it still remain unclear. The observed results appear to be a result of fatty acid and lipid composition of the diet which was also reflected in fatty acid profiles of unfertilized eggs, but few studies have investigated this topic for the spotted wolffish. No chemical analysis was performed on sperm, which should also be investigated in future studies. The current experiment investigated eggs from only 5 females from each group, and even though the results for egg survival was correlated to the fatty acid composition of unfertilized eggs, genetic variance cannot be excluded of possible explanation of the observed results. This is the first study performed on broodstock nutrition on the spotted wolffish, and further studies should focus on investigating optimum concentrations of the respective ingredients to develop a species-specific diet with the purpose of optimizing survival throughout the earliest life stages.

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8 Appendixes

Appendix 1. Chemical composition and content of the experimental low-fat diet. Content given as %.

Ingredient	Content (%)
Fishmeal LT70	15.00
Fish protein hydrolysate	7.50
Squid meal	30.00
Krill meal	5.00
Blue mussel meal	12.50
Wheat gluten	5.00
Wheat meal	9.50
Potato starch	5.23
Vitamin and mineral premix	1.50
Vitamin C35	0.02
Vitamin E50	0.15
Betaine HCI	0.10
Antioxidant	0.25
Sodium propionate	0.10
Monoammonium phosphate	2.00
SelPlex 600 – Se yeast (ALLTECH)	0.10
Nucleotides (Nucleoforce)	0.250
L-Histidine	0.15
L-Taurine	0.25
L-Glutamine	0.25
Glycine	0.10
Soy lecithin	0.50

Algae oil	4.30
Arachidonic acid 40%	0.250
Total	100.00

Appendix 2. Abbreviations for chemicals used in the commercial extender.

Abbreviation	Chemical
NaCl	Natrium Chloride
$CaCl_2$	Calcium Chloride
$KHCO_2$	Potassium Formate
$MgSO_4$	Magnesium Sulphate

Appendix 3. Abbreviations used for chemicals and methods of lipid analysis.

Abbreviation	Chemical
MeOH	Methanol
BHT	Butylated hydroxytoluene
DCM	Methylene chloride
KCl	Potassium chloride
N2	Nitrogen
H_2SO_4	Sulfuric Acid
$KHCO_3$	Potassium bicarbonate
GC-FID	Gas Chromatography Flame Ionization

Appendix 4. Full fatty acid profile of unfertilized spotted wolffish eggs from females reared at different dietary treatments. Overview of lipid content in unfertilized eggs for both diet groups. Given as g/100g lipid (%), means ±SD. Significant differences are highlighted in bold (p<0.05).

Lipid	HFD	LFD
14:0 FA	1.53 ± 0.1	1.02 ± 0.06
16:0 FA	12.77 ± 0.64	13.90 ± 0.35
17:0 FA	0.24 ± 0.01	0.25 ±0.02
18:0 FA	4.90 ± 0.37	4.58 ± 0.25
16:1 n-7	4.02 ± 0.37	4.22 ± 0.21
18:1 n-7	2.54 ± 0.09	2.29 ± 0.08
18-1 n-9	22.8 ±1.20	16.4 ± 1.20
20:1 n-9	1.18 ±0.05	0.99 ± 0.10
18:2 n-6	7.62 ± 0.32	4.40 ± 0.53
20:4 n-6	**1.34 ± 0.06**	**2.80 ± 0.11**
18:3 n-3	1.30 ± 0.05	0.68 ± 0.06
18:4 n-3	0.66 ± 0.08	0.45 ± 0.14
20:4 n-3	**1.05 ± 0.15**	**1.58 ± 0.12**
20:5 n-3	**10.40 ± 0.40**	**12.57 ± 0.11**
22:5 n-3	1.35 ± 0.09	1.71 ± 0.12
22:6 n-3	**20.59 ± 1.31**	**25.74 ± 0.17**
n-3: n-6	3.87 ± 1.05	5.76 ±0.08
EPA + DHA	**30.99 ± 1.18**	**38.31 ± 0.24**
Sum SFA	21.40 ± 0.40	22.30 ± 0.70
Sum MUFA	**31.60 ± 1.90**	**26.0 ± 1.3**
Sum PUFA	**46.00 ± 1.60**	**51.70 ± 0.9**
Sum n-3 FA	**35.80 ± 0.4**	**43.30 ± 0.4**
Sum n-6 FA	**9.20 ± 0.3**	**7.50 ± 0.5**
Sum n-9 FA	**25.10 ± 1.5**	**18.3 ± 1.1**

Appendix 5. Overview over results from spearmans correlation test. Significant correlations are highlighted in bold (p<0.05).

Parameter	*p*-value	R-value
VCL ~ pH	0.04	0.54
MOT ~ pH	0.125	0.42
Spermatocrit ~ Volume	0.09	0.46
Female weight ~ Fecundity	0.56	0.20
EPA ~ Survival pre-hatch	0.025	0.70
EPA:AA ~ Survival pre-hatch	0.019	-0.72
DPA ~ Survival pre-hatch	**0.04**	**0.64**
DHA ~ Survival pre-hatch	**0.017**	**0.73**
Abnormal cleaving~ Deformity rate	0.60	-0.39
Abnormal cleaving ~Survival pre-hatch	0.38	0.35
Length day 0 ~ Length Day 50	**0.006**	**0.79**
Oleic acid ~ Survival pre-hatch	0.006	-0.79
AA ~Survival pre-hatch	**0.025**	**0.70**
AA~Hatching rate	0.053	0.62
Oleic acid ~ Survival pre-hatch	**0.01**	**-0.78**
16:0 ~ Survival pre-hatch	**0.026**	**0.70**
Omega 3 ~ Hatching rate	0.07	0.60
Omega 3 ~ Survival pre-hatch	**0.04**	**0.64**
AA ~ Length day 0	**0.01**	**0.74**
EPA ~ Length day 0	**0.04**	**0.65**