Aquaculture Research

Aquaculture Research 2015, **46**, 1924–1936 doi: 10.1111/are.12347

Growth performance, survival and body composition of southern flounder *Paralichthys lethostigma* larvae fed different formulated microdiets

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Abstract

Three experiments were conducted to evaluate the effects of micro-bound diets (MBD) on southern flounder larvae. In experiment 1, four MBDs were formulated with different protein sources as follows: MBD 1: herring meal, MBD 2: menhaden meal, MBD 3: menhaden and squid meal; MBD 4: menhaden, squid and herring meal. In experiment 2, four MBDs were formulated as follows: MBD 5: menhaden, squid and herring meal; MBD 6: menhaden, squid, herring and attractants; MBD 7: menhaden, squid, herring and casein, and MBD 8: menhaden, squid, herring, casein and attractants. In experiment 3, three groups were maintained as follows: Group 1: live feed; Group 2: co-fed with MBD 6; and Group 3: MBD 6. In experiment 1 on 35 dph, survival and body weight (BW) of the fish fed MBD 4 was significantly higher than the MBDs 1 and 2. In experiment 2 on 34 dph, fish fed MBD 6 had significantly higher BW than the commercial microdiets. In experiment 3 on 21 dph, fish receiving only MBD had significantly lower survival than the other groups. Growth, survival and larval fatty acid composition suggested that co-feeding MBD 6, a mixture of marine protein sources plus attractants was more effective than the other MBDs.

Keywords: live feed, co-feeding, microdiets, southern flounder, larvae

Introduction

Southern flounder *Paralichthys lethostigma* has great promise for aquaculture, with high market value and global demand, declining natural populations and a unique ability to grow well in fresh, brackish or full-strength sea water (Daniels, Losordo & Watanabe 2006). The culture technologies are being transferred to commercial users in southeastern United States to establish southern flounder as a viable aquaculture industry in the United States The spawning technique of southern flounder broodstock (Watanabe, Carroll & Daniels 2001; Watanabe, Woolridge & Daniels 2006) and the basic environmental and nutritional conditions for successfully raising larvae through metamorphic stages have been delineated, including temperature (Van Maaren & Daniels 2001), salinity (Moustakas, Watanabe & Copeland 2004; Mangino & Watanabe 2006), light intensity (Henne & Watanabe 2003), photoperiod (Moustakas *et al.* 2004) and water turbulence (Mangino & Watanabe 2006). Economic analysis of a southern flounder recirculating aquaculture system grow-out operation showed that fingerling costs are among the most important components of financial performance, representing 24-29% of total production costs (Dumas, Watanabe, Daniels, Losordo & Yates 2006). Development of a less expensive formulated diet with full nutrition and good physical and biochemical properties for partially or completely

replacing live food will decrease production costs for southern flounder enterprises.

Live feeds such as rotifers *Brachionus* sp. and brine shrimp *Artemia* are considered to be essential for the production of marine fish juveniles (Baskerville-Bridges & Kling 2000; Sorgeloos, Dhert & Candreva 2001). However, high costs and labour requirements along with the highly variable nutritional value of live feeds and the unreliability of mass cultures associated with the production of live feeds illustrate the need to find viable alternatives (Holt 1993; Baskerville-Bridges & Kling 2000; Salhi & Bessonart 2013). Moreover, the provision of live food is characteristically plagued with variable supply and nutritional quality. Consequently, a great deal of interest has been generated to develop an off-the-shelf artificial larval microdiet as an economic live food alternative. Moreover, microdiets offer the opportunity to introduce nutrients into the larvae that are not available in the live feed (Rosenlund, Stoss & Talbot 1997). Minimizing or eliminating the use of live feeds is economically advantageous for marine finfish hatcheries (Baskerville-Bridges & Kling 2000). Person-Le Ruyet, Alexandre, Thebaud and Mugnier (1993) found that, by starting the weaning process of European sea bass *Dicentrachus labrax* onto microdiets at 20 day post hatching (dph) instead of 35 dph, up to 80% of live feed costs were reduced during weaning, 50% of the total cost of 3-month-old juveniles.

Artificial microdiets and their dietary values for several species have been investigated for more than 25 years. Much work has been conducted to rear larval fish with microdiets (Teshima, Kanazawa & Sakamoto 1982; Kanazawa, Koshio & Teshima 1989; Jones, Kamardin & Vay 1993; Langdon 2003; Curnow, King, Bosmans & Kolkovski 2006; Tang, Chen & Wu 2010). Apart from freshwater species, however, most attempts to rear larval marine fish with only microdiets have not been completely successful in terms of survival, growth and stress tolerance. Total replacement of live food with microdiets has been accomplished in shrimp, but not in marine fish (Cahu & Zambonino-Infante 2001). In developing microdiets suitable for early weaning, the food particles should be water stable, palatable, nutritionally complete and digestible by the larvae (Yufera, Pascual & Fernandez-Diaz 1999). However, the replacement of live food in the larval rearing of marine fish with a formulated diet has been a challenge to aquaculture, nutrition and feed formulation scientists.

Zein bound microdiets were used successfully in the larval rearing of red sea bream and Japanese flounder (Kanazawa *et al.* 1989; Teshima, Ishikawa & Koshio 2000; Teshima, Koshio, Ishikawa, Alam & Hernandez 2004). Zein is a high molecular weight (38 000 Da), non-toxic, edible protein with amphoteric and thermoplastic properties. Zein is soluble in 40–90% alcohol, making preparation of zein microparticles less toxic compared with preparation methods for other particle types that involve the use of toxic solvents. Ethanol can also be easily removed by evaporation or spray-drying to form zein-bound complex particles. Furthermore, amino acids and many other low molecular water-soluble materials are significantly less soluble in ethanol than pure water, reducing the leakage loss of core materials during particle preparation (Langdon, Clack & Önal 2007).

Macronutrient requirements and utilization of alternative protein sources in juvenile southern flounder diets have been reported (Alam & Watanabe 2005; Alam, Watanabe & Daniels 2009; Alam, Watanabe, Myers, Rezek, Carroll & Longfellow 2011). However, experimental data regarding formulated microdiets for rearing larval southern flounder is limited. More than 80% of the southern flounder larvae complete metamorphosis by day 31 (Daniels & Watanabe 2003). Therefore, it is important to feed a high quality microdiet to larvae stage for better performance and survival. In this study, the effectiveness of various dietary protein sources in promoting growth and survival of southern flounder larvae was determined. The objectives were to evaluate zein bound microdiets (MBD) formulated with different protein sources supplemented with or without attractants as feed for southern flounder larvae.

Materials and methods

Experimental design and diets

Experiment 1

Four different iso-nitrogenous (54% crude protein) and iso-lipidic (12% lipid) microbound diets (MBD) were formulated and prepared with different high quality protein sources as follows (Table 1): MBD 1: herring meal, MBD 2: menhaden meal, MBD 3: menhaden and squid meal; MBD 4: menhaden, squid and herring meal. All MBDs contained 5% krill meal and 1% attractants (alanine, glycine, taurine and betaine). Vitamin (5%) and minerals

Table 1 Composition of microbounds diets (MBDs) (g 100 g^{-1} dry basis) (experiment 1)

Ingredients	MBD 1	MBD 2	MBD 3	MBD 4
Herring meal*	64	0	0	23
Krill meal†	5	5	5	5
Squid meal‡	0	0	32	20
Menhaden meal§	0	68	30	20
Zein¶	10	10	10	10
Menhaden fish oil	3.5	2.0	2.5	2.5
Soybean lecithin	2.5	2.5	2.5	2.5
n-HUFA	0.5	0.5	0.5	0.5
Wheat starch	3	1	3.5	2.5
Vitamin mix	5	5	5	5
Mineral mix	5	5	5	5
Attractants‖	1	1	1	1
Cellulose	0.5	0	3	3
Total	100	100	100	100
Proximate composition in diets				
Protein	54.8	54.2	54.6	54.8
Lipid level	11.8	12.3	12.2	11.7

*Protein 67%, lipid 7.5%.
†Protein 60%, lipid 10%.
‡Protein 75%, lipid 10%.
§Protein 60%, lipid 10%.
¶Protein 90%.
‖Attractants; Alanine, Betaine, Glycine and Taurine (each 0.25%).

Table 2 Composition of microbound diets (MBDs) (g 100 g^{-1} dry basis) (experiment 2)

Ingredients	MBD 5	MBD 6	MBD 7	MBD 8	Otohime*
Herring meal	23	23	10	10	
Krill meal	5	5	5	5	
Squid meal	20	20	20	20	
Menhaden meal	20	20	20	20	
Casein	0	0	10	10	
Zein	10	10	10	10	
Menhaden fish oil	5	5	6	6	
Soybean lecithin	2.5	2.5	2.5	2.5	
n-HUFA	0.5	0.5	0.5	0.5	
Vitamin	5	5	5	5	
Mineral	5	5	5	5	
Taurine	0	0.25	0	0.25	
Alanine	0	0.25	0	0.25	
Glycine	0	0.25	0	0.25	
Betaine	0	0.25	0	0.25	
Cellulose	1	0	3	2	
Wheat Starch	3	3	3	3	
Total	100	100	100	100	
Protein%	54.81	54.81	55.1	55.1	51
Lipid%	14.22	14.22	14.2	14.25	11
Energy kj g^{-1} diet	15.01	15.01	15.07	15.07	

*Reed Mariculture Inc., Campbell, CA, USA.

(5%) were the same for all diets. Menhaden oil and soybean lecithin were added as lipid sources and menhaden fish oil was adjusted to maintain all MBDs iso-lipidic. N 3-HUFA was also added to meet the DHA and EPA requirements for flatfish larvae (Teshima *et al.* 2004). Wheat starch was added as a carbohydrate source and adjusted to maintain all MBDs iso-caloric.

Experiment 2

Four MBDs were formulated and prepared with a mixture of high quality protein sources and compared with a commercially available microdiet (Otohime, Japan). The four diets formulated were as follows (Table 2): MBD 5: menhaden, squid, herring and krill meal, MBD 6: menhaden, squid, herring meal, krill meal and attractants, MBD 7: menhaden, squid, herring, krill meal and casein, MBD 8: menhaden, squid, herring, krill meal, casein and attractants and commercial microdiet: Otohime (Reed Mariculture Inc., Campbell, CA, USA).

The MBDs were formulated to contain about 55% protein and 14% lipid. All MBDs contained 5% krill meal, 20% menhaden meal and 20% squid meal, similar to the MBD 4 in Experiment 1. MBD 5 was formulated as MBD 4 in Experiment 1, but without attractants, whereas MBD 6 was the same as MBD 4 (with 1% attractants). MBD 7 was formulated with 10% casein and 10% herring meal in addition to menhaden meal (20%), squid meal (20%) and 5% krill meal. The amounts and protein sources in MBD 8 were the same as MBD 7, but 1% attractants was added in MBD 8. One per cent attractants (alanine, taurine, glycine and betaine, 0.25% of each) was used in MBD 6 and 8, respectively, as reported for Japanese flounder *Paralichthys olivaceus* (Teshima *et al.* 2004). Differences in the levels of attractants and fish oil among diets were adjusted with cellulose. Menhaden fish oil and soybean lecithin were used as lipid sources and vitamin and mineral premix were used in equivalent amount in all MBDs.

Preparation of MBDs

Before preparing the MBDs, all protein sources were sieved (125–250 μm mesh). The MBDs were

prepared (Teshima *et al.* 1982; Kanazawa *et al.* 1989) and then ground and sieved into particles ranging from 125 to 550 μm. The lipid sources (menhaden fish oil, soybean lecithin and n-HUFA) were mixed with 300 mL of 60% ethanol in an electric blender. The zein and the remaining ingredients were added and mixed thoroughly until all the ethanol evaporated, and the resulting dough was freeze dried for 48 h. Dry diets were crumbled into particles, then sorted with sieves (125, 250, and 355 μm mesh) to obtain the desired particle sizes. MBDs were stored at −24°C.

Flounder spawning

Southern flounder embryos and larvae were produced by induced spawning of photothermally conditioned broodstock held at the UNCW Aquaculture Facility (Watanabe *et al.* 2006). Embryos were stocked in 300-L larval rearing tanks. Newly-hatched larvae were fed rotifers and *Artemia* from 3 to 10 dph until the start of the MBD feeding experiments. Rotifers were grown in a continuous culture system using *Nannochloropsis oculata* paste (Reed Mariculture, Campbell, CA, USA). Rotifers and *Artemia* nauplii were enriched (Algamac 2000, BioMarine Aquafauna, Hawthorne, CA, USA), to boost highly unsaturated fatty acids (HUFA).

Feeding trials with MBDs

Experiment 1

For feeding experiments, larvae were transferred from the stocking tank (300 L) to 12, 200-L experimental tanks containing 150 L seawater at ambient temperature (22–23°C) and supplied with diffused aeration under a 12 h : 12 h light/dark photoperiod. Rearing conditions followed published hatchery protocols for southern flounder (Daniels & Watanabe 2003; Henne & Watanabe 2003; Moustakas *et al.* 2004; Mangino & Watanabe 2006). Triplicate tanks were maintained for each treatment. Each experimental tank was stocked with 1,500, 14 day post hatching (dph) larvae (10 L^{-1}) and maintained on four dietary treatments (Table 1). Fish were co-fed (Table 3) live feed (1/2 live food, rotifer and *artemia*, two times a day) and MBD (0.5 mg/fish/day) four times per day (8:30 AM; 11:30 AM; 14:30 PM and 16:30 PM) as recommended for other marine fish larvae (Teshima *et al.* 2004). MBDs were given in each feeding period using a small spoon and blowing the feed into the water. MBD particle sizes were adjusted according to fish mouth sizes based on visual observations (Table 3).

Table 3 Feeding protocols of live feeds and MBDs

Experiments 1 and 2:

1	5	10	15	20	25	31	35

Day post-hatching (dph)

Rotifer — *Artemia*

MBD (125–250 μm) — MBD (250–355 μm) — MBD (355–500 μm)

Experiment 3:

Group 1: live feed

Group 2: 50% live feed + MBD

Group 3*: only MBD

* In Group 3, 14 dph-17 dph = 25% live feed decreased daily, 18 dph-25 dph: only MBD.

In all three Expts:

Feeding of live feed: Daniels & Watanabe (2003)

Feeding rate of MBD: 1- 6 mg fish/d (Teshima et al. 2004)

Feeding frequency of MBD: 5 times/day (8:30, 10:30, 13:00, 14:30, 16:30 h)

Experiment 2

Larvae were reared to 10 dph in 300-L tanks. To begin the feeding trial, larvae (10 dph) were transferred to 30-L tanks at a density 27 L^{-1}, or approximately 800 larvae per tank. The larvae were co-fed (Table 3) live food (1/2 half of live feed, rotifer and *artemia*) and the four MBDs and a commercial microdiet Otohime with four replicate tanks per treatment. The test and commercial MBDs were provided five times per day (8:30 AM; 10:30 AM; 13:00 PM; 14:30 PM and 16:30 PM) as in experiment 1 (Table 3).

Experiment 3

Larvae were reared to 10 dph in 300-L tanks. Treatment diets were fed beginning on 11 dph in 30-L tanks stocked at a density of 30 L^{-1}, or 1000 larvae/tank. Five replicate tanks were maintained for each diet treatment. Based on best growth performance in Experiments 1 and 2, MBD 6 was selected for this trial. Three feeding groups were maintained: Group 1: only live feed (rotifer

and *Artemia,* 2 times a day) until 25 dph, Group 2: ½ live feed and ½ MBD 6 (co-feeding) until 25 dph, and Group 3: only MBD 6 (live feed reduced 25% each day, with the amount of microdiets increased proportionally) until 21 dph. MBD 6 was fed to groups 2 and 3 at five times per day (08:30 AM, 11:30 AM, 13:00PM; 14:30 PM, and 16:30 PM) as in Experiment 1 (Table 3).

Water quality parameters

Seawater for larval culture was pumped from the Atlantic Intracoastal Waterway adjacent to Wrightsville Beach, and passed through a 1-μm filter and treated with UV light before use. Total ammonia nitrogen (TAN) (mg L^{-1}) was monitored weekly (HACH DR 850; Hach, Loveland, CO, USA). Temperature, salinity, dissolved oxygen and pH (YSI 55, YSI Incorporated, Yellow Springs, OH, USA) of the water were monitored every 2 days. Water quality parameters for Experiments 1, 2 and 3 were maintained at optimal levels (Daniels & Watanabe 2003). Light intensity at the water surface of each tank was measured using a light meter (Extech Instruments, Waltham, MA, USA). Airflow (40–60 mL min^{-1}) to each tank was monitored using a flow meter (Cole-Parmer Instrument, Vernor Hills, IL, USA) and adjusted as needed. Tanks' surfaces were skimmed daily with paper towels to remove oil films, if any.

Growth performance and survival determination and sample collection

Performance of fish on test microdiets was assessed by final body weight (FBW), total length (TL), and survival (%). Measurements of TL were conducted using a microscope with 20 fish randomly collected from each tank. For body weight and length, initially 200 and then 40 from each tank were collected at each sampling, rinsed with freshwater and blotted to remove excess water, and then wet weights (mg $fish^{-1}$) were measured by weighing fish with an electronic microbalance. The larvae were freeze dried and dry weights were measured using a microbalance and then stored at −80°C for proximate analysis.

Leaching test of MBDs

Leaching rates were determined by adding approximately 100 mg (weighed to four decimal places) of each diet into a beaker containing 50 mL of sea water at room temperature (20°C). The samples were stirred using a small stirrer to keep the MBD particles suspended in the water column for the duration of the leaching process. Pre-weighed freeze-dried filter paper (FP) was used to filter the MBDs. The filtering process was timed so that the water was removed from the filter funnel within 10 min ± 5 s. Distilled water was used to wash the sample at the end of the filtering process to remove salt from the MBD. The MBD and FP were then freeze dried and the leaching losses of dry matter from the MBD were determined using a calculated initial dry weight (DW) of the MBD and FP and final DW of MBD and FP, as follows:

$$\text{Leaching losses} = \frac{\text{Initial (MBD. DW + FP. DW)} - \text{Final (MBD. DW + FP. DW)}}{\text{Initial (MBD. DW)}} \times 100\%$$

Fatty acid composition

Fatty acid composition of the larval whole bodies was determined by first extracting total lipids into chloroform: methanol (Folch, Lees & Sloane-Stanley 1957). Lipid fatty acids were converted to their methyl esters (FAMEs) for GC analysis by refluxing the concentrated lipid sample in 1.0 mL of 0.5 M NaOH/MeOH for 30 min., followed by addition of 1.5 mL of boron trifluoride-methanol (BF_3) and refluxing for an additional 30 min. The FAMEs were extracted into hexane, concentrated and redissolved in 1 mL of chloroform. GC analysis was performed on a HP-6890 (HP, Palo Atto, CA, USA) Gas Chromatograph using a 25 m × 0.25 μm HP-5 capillary column with FID detection (Department of Chemistry and Biochemistry, UNCW). Helium was used as the carrier gas. The column temperature profile was: 195°C, hold for 8 min, ramp to 270 at 15°C min^{-1} and hold at 270°C for 2 min. FAME peaks were integrated using the HP Chemstation software (HP) package and individual FAMEs were identified by comparison of retention times to standards: GLC-84 (Nu-Check Prep. Inc., Elysian, MN, USA) as well as individual standards of stearidonic, eicosapentaenoic and arachidonic acid methyl esters (Sigma-Aldrich, St. Louis, MO, USA). FAMEs from all

samples were quantified using their peak areas compared to the peak area of the C19 : 0 internal standards.

Chemical analyses and statistical evaluation of data

Proximate analysis of MBDs and larval whole bodies were conducted following the method of AOAC (Association of Official Analytical Chemists) (2000). Growth data were analyzed by one-way analysis of variance (JMP ver. 7; SAS, Cary, NC, USA). Per cent survival data were arc-sine transformed before analysis, and significant differences among dietary treatments were tested by Tukey's Kramer test. Probabilities of $P < 0.05$ were considered significant (Kramer 1956).

Results

Water quality parameters

In Experiment 1, water quality parameters were as follows: temperature 20.5 ± 0.56 C (mean ± SD), pH 8.3 (8.2–8.4) salinity 33.5 ± 0.30 g L^{-1}, dissolved oxygen 7.4 ± 0.1 mg L^{-1}, ammonia 0.26 ± 0.01 mg L^{-1} and nitrate 0.12 ± 0.01 mg L^{-1}. Water quality parameters for experiment 2 were temperature 21.1 ± 0.61 C, pH 8.3 (8.2–8.4), salinity 33.7 ± 0.40 g L^{-1}, dissolved oxygen 6.8 ± 0.1 mg L^{-1}, ammonia 0.33 ± 0.01 mg L^{-1} and nitrate 0.10 ± 0.01 mg L^{-1}. In experiment 3, water quality parameters were temperature 20.7 ± 0.7 C, pH 8.2 (8.1–8.4), salinity 34.5 ± 0.30 g L^{-1}, dissolved oxygen 6.9 ± 0.1 mg L^{-1}, ammonia 0.25 ± 0.01 mg L^{-1} and nitrate 0.1 ± 0.01 mg L^{-1}. Light intensity at the water surface of each tank was 250 ± lx.

Growth performance and survival

Table 4 shows the total length (TL), final body weight (FBW) and survival during the 20-day feeding trial of larval southern flounder fed different MBDs in Experiment 1. No significant differences in TL were observed among the fish fed different MBDs (Table 4). The best FBW (dry weight basis) was obtained on MBD 3 (966 μg) and on MBD 4 (963 ± 34 μg), which were significantly ($P < 0.05$) higher than MBD 1 (696 ± 47 μg, average ± SEM) and MBD 2 (747 ± 29 μg). The highest survival was found for the fish fed MBD 4 (84%), significantly higher than those fed the other MBDs (68–75%).

Performance of southern flounder larvae fed different MBDs in Experiment 2 is shown in Table 5. At 34 dph, larvae fed the MBDs and commercial microdiets were not significantly different in TL (8.4–9.0 mm). FBW of fish fed MBD 6 (1134 μg) was significantly higher than in fish fed commercial microdiets (780 μg), but not significantly different from the MBDs 5, 7 and 8 (880–909 μg). Final survival was not significantly different among diets (23.4–31%).

The growth performances of larvae in experiment 3 are shown in Table 6. On 18 dph, there were no significant differences among treatments in TL (5.24–5.4 mm), FBW (117–122 μg) and survival (58.7–66.2%). On 21 dph, FBW of larvae fed only MBD 6 (104 μg) was significantly lower than larvae fed live feed (LF) or co-fed live feed and MBD (CO) (154–176 μg). However, on 21 dph, the LF and CO treatments did not show significant differences in TL (5.67–5.82 mm), FBW (154–176 μg) and survival (36.3–43.5%). On 21 dph, survival of larvae fed only MBD 6 (22.1%) was significantly lower than in the LF or

Table 4 Total length (TL), final body weight (FBW) and survival (%) of the southern flounder in the 20-day feeding trial (15 dph-35 dph, experiment 1). Values are means ± SEM of triplicate tanks. Means with different letters in the same column differ significantly ($P < 0.05$)

Diets	TL (mm)	FBW (μg/fish dry weight)	Survival (%)
MBD 1	7.1 ± 0.24	696 ± 47^{a}	68 ± 1.6^{a}
MBD 2	7.2 ± 0.08	747 ± 29^{a}	73 ± 1.2^{a}
MBD 3	7.4 ± 0.25	966 ± 21^{b}	75 ± 1.0^{a}
MBD 4	7.5 ± 0.13	963 ± 34^{b}	84 ± 1.2^{b}

Table 5 Total length (TL), final body weight (FBW) and survival (%) of the southern flounder after the 24-day feeding trial (10 dph–34 dph, experiment 2). Values are means ± SEM of triplicate tanks. Means with different letters in the same column differ significantly ($P < 0.05$)

MBDs	TL (mm)	FBW (μg/fish dry weight)	Survival (%)
MBD 5	8.8 ± 0.13	938 ± 64^{ab}	26.7 ± 2.06
MBD 6	9.0 ± 0.12	1134 ± 108^{a}	26.0 ± 1.5
MBD 7	9.0 ± 0.10	909 ± 74^{ab}	24.7 ± 0.85
MBD 8	8.4 ± 0.11	880 ± 55^{ab}	23.4 ± 1.01
Otohime	8.6 ± 0.12	780 ± 73^{b}	31.0 ± 3.8

Table 6 Total length (TL), final body weight (FBW) and survival (%) of the southern flounder fed live feed (LF), co-fed live feed and microbound diets (CO), and microbound diets (MBD 6) after the 14-day feeding trial (11 dph–25 dph, experiment 3). Values are means ± SEM of triplicate tanks. Means with different letters within column and age group indicates significant differences among treatments ($P < 0.05$)

Group	TL (mm)	FBW (μg/fish dry weight)	Survival
18dph			
LF	5.24 ± 0.3	122 ± 11	58.7 ± 4.8
CO	5.40 ± 0.3	117 ± 11	60.3 ± 14.5
MBD 6	5.24 ± 0.06	117 ± 7	66.2 ± 11.7
21dph			
LF	5.67 ± 0.11	154 ± 20[a]	43.5 ± 8.3[a]
CO	5.82 ± 0.06	176 ± 19[a]	36.3 ± 12.1[a]
MBD 6	6.06 ± 0.21	104 ± 4[b]	22.1 ± 4.3[b]
25dph			
LF	6.44 ± 0.17	449 ± 45	20.2 ± 11
CO	6.22 ± 0.24	438 ± 178	19.5 ± 8
Only MBD 6		no survival by 25 dph	

CO treatments. On 25 dph, no significant differences were found between LF and CO treatments in TL (6.22–6.44 mm), FBW (438–449 μg) and survival (19.5–20.2%). In experiment 3, by 24 dph, survival in larvae fed only MBD 6 was 0%.

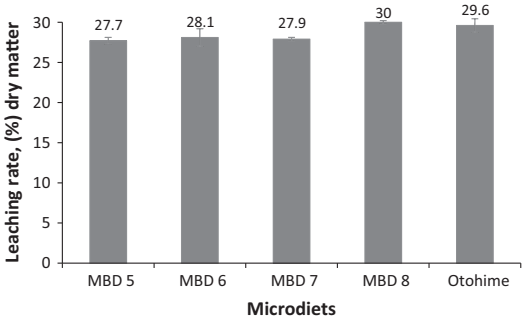

Figure 1 Leaching rates (% dry matter) for microdiets after 10 min immersion in seawater (experiment 2).

Table 7 Final proximate composition of whole body of southern flounder larvae (% wet wt) in Experiments 1 and 2. Data are mean ± SEM (n = 3)

Diets	Moisture	Crude protein	Lipid
Experiment 1			
MBD 1	85.3 ± 0.33	7.86 ± 0.23	2.07 ± 0.09
MBD 2	85.7 ± 0.37	7.72 ± 0.13	1.76 ± 0.13
MBD 3	85.8 ± 0.33	7.70 ± 0.17	1.85 ± 0.05
MBD 4	85.7 ± 0.30	8.03 ± 0.18	1.77 ± 0.12
Experiment 2			
MBD 5	84.6 ± 1.07	8.25 ± 0.37	2.75 ± 0.06
MBD 6	84.6 ± 1.1	8.56 ± 0.36	2.91 ± 0.03
MBD 7	85.7 ± 1.07	8.13 ± 0.29	2.85 ± 0.06
MBD 8	85.2 ± 0.9	8.60 ± 0.26	2.86 ± 0.03
Otohime	84.7 ± 1.9	8.76 ± 0.37	2.81 ± 0.06

Leaching of dry matter

The leaching losses of dry matter of MBDs 5, 6, 7, 8 and commercial microdiets are shown in Fig. 1. MBDs and commercial microdiets leaching resulted in losses of 27.8–30.0% of dry matters after 10 min in sea water with no significant differences among the diets (Fig. 1).

Proximate composition of the whole bodies

In experiments 1 and 2, the proximate analysis of the whole bodies of larvae after the feeding trials showed that the moisture, protein and total lipid levels were not significantly different among the MBDs (Table 7).

Fatty acid composition of fish larvae

Fatty acid composition of the southern flounder larvae fed the different microdiets in experiment 2 are presented in Table 8. Linoleic acid (18 : 2n-6) was significantly lower ($P < 0.05$) in larvae fed commercial microdiets (2.87% total fatty acids) than in the formulated MBDs (3.63–4.02), but there were no significant differences among these formulated MBDs. However, EPA (20 : 5n-3) content in larvae fed Otohime (4.85) was significantly higher than in larvae fed MBDs 5 (3.89), 6 (3.90) and 7 (3.88), but not MBD 8 (4.46). DHA (22 : 6n-3) (16.9–17.4) and total n-3 PUFA (35.5–37.3) content in fish larvae were not significantly different between the larvae fed commercial microdiets and MBD 6. N-3 PUFA were significantly higher in larvae fed commercial microdiets than in those fed MBD 5, 7 and 8, but not with MBD 6. However, total n-6 PUFA were significantly higher in larvae fed the formulated MBDs (9.02–9.48) than the larvae fed commercial microdiets (7.18).

Table 8 Fatty acid composition of southern flounder larvae fed different microdiets (% of total fatty acids). Data are mean ± SEM (n = 3) (experiment 2). Means with different letters in the same column differ significantly ($P < 0.05$)

Fatty acids	MBD 5	MBD 6	MBD 7	MBD 8	Otohime
14 : 1	1.63 ± 0.24	1.37 ± 0.06	1.34 ± 0.05	1.47 ± 0.01	1.70 ± 0.03
16 : 1n-7	2.66 ± 0.16	2.54 ± 0.06	2.57 ± 0.11	2.73 ± 0.03	2.73 ± 0.04
16 : 0	20.3 ± 0.47	19.8 ± 0.20	19.8 ± 0.42	19.8 ± 0.42	19.5 ± 0.17
18 : 2n-6	3.76 ± 0.21^{a}	3.64 ± 0.08^{a}	3.63 ± 0.06^{a}	4.02 ± 0.09^{a}	2.87 ± 0.02^{b}
18 : 3n-3	11.5 ± 0.18^{ab}	11.6 ± 0.16^{ab}	11.3 ± 0.12^{b}	11.5 ± 0.12^{ab}	12.0 ± 0.11^{a}
18 : 1n-11	4.53 ± 0.09	4.10 ± 0.41	4.59 ± 0.08	4.63 ± 0.01	4.66 ± 0.09
18 : 0	10.2 ± 0.26^{a}	10.2 ± 0.21^{a}	10.4 ± 0.04^{a}	9.99 ± 0.16^{a}	8.84 ± 0.14^{b}
19 : 0	8.12 ± 1.09	7.68 ± 0.07	7.37 ± 0.51	7.47 ± 1.18	7.32 ± 0.04
20 : 4n-6	5.26 ± 0.01^{a}	5.61 ± 0.19^{a}	5.65 ± 0.02^{a}	5.46 ± 0.18^{a}	4.31 ± 0.04^{b}
20 : 5n-3	3.89 ± 0.04^{b}	3.90 ± 0.18^{b}	3.88 ± 0.05^{b}	4.46 ± 0.07^{a}	4.85 ± 0.12^{a}
20 : 5n-6	3.65 ± 0.33^{ab}	3.50 ± 0.21^{ab}	4.01 ± 0.03^{a}	3.67 ± 0.17^{ab}	2.90 ± 0.09^{b}
20 : 1	1.84 ± 0.03^{b}	1.81 ± 0.03^{b}	1.81 ± 0.01^{b}	1.83 ± 0.04^{b}	2.61 ± 0.02^{a}
22 : 6n-3	16.4 ± 0.16^{ab}	16.9 ± 0.49^{ab}	16.8 ± 0.11^{ab}	15.9 ± 0.39^{b}	17.4 ± 0.05^{a}
22 : 5n-3	2.85 ± 0.03	3.10 ± 0.11	2.96 ± 0.05	3.13 ± 0.07	3.03 ± 0.05
SUM of n-3 PUFA	34.6 ± 0.2^{a}	35.5 ± 0.04^{ab}	34.9 ± 0.02^{a}	34.9 ± 0.63^{a}	37.3 ± 0.16^{b}
SUM of n-6 PUFA	9.02 ± 0.21^{a}	9.26 ± 0.13^{a}	9.29 ± 0.05^{a}	9.48 ± 0.25^{a}	7.18 ± 0.03^{b}

Discussion

The digestion of dietary proteins in larval fish has been known to differ from that in juveniles (Kolkovski 2001). In many larval marine fish lacking a functional stomach, proteolytic enzyme activity is not fully developed (Langdon 2003). Quality MBD for larval aquatic animals are needed to meet the following requirements: nutritionally well-balanced, high water-stability (minimal leaching of nutrients), high digestibility and proper suspension in the water column (Bengtson 1993; Teshima *et al.* 2000; Izquierdo & Fernandez-Palacios 1997). Therefore, this study was designed to see the effects of different protein sources on the performance of southern flounder larvae.

'Co-feeding' weaning protocols as used in Experiments 1 and 2 simultaneously use inert and live diets to allow a fast and efficient change-over period onto dry microdiets from live feed (Daniels & Hodson 1999; Koven, Kolkovski, Hadas, Gamsiz & Tandler 2001). This method has been found to achieve higher growth and survival than feeding either live feeds or MDs on their own (Kolkovski 2001; Wang, Hu, Wang & Cao 2009).

In experiment 1, larvae fed MBD 3 and MBD 4 containing both fish meal and squid meal as protein sources showed better growth performance than those fed MBDs containing only one of these sources after 20 days of feeding. Fish meal and squid powder were found to have high nutritional value as protein sources for barramundi *Lates calcarifer* larvae by virtue of a synergistically favourable amino acid profile and moderate to high digestibility (Nankervis & Southgate 2006; Saen de Rodriganez, Gander, Alaiz & Moyano 2011). Higher dry weight and survival of larvae (Table 4) at the end of experiment 1 clearly indicated that the MBD containing a combination of fish meal, squid meal, herring meal and krill meal (MBD 4) was superior to MBDs without squid meal (MBDs 1 and 2).

Better growth performance of southern flounder larvae in terms of final dry body weight of MBD 3 and 4 could indicate that protein quality and digestibility in these MBDs were higher than in MBD 1 and 2. The quality of dietary protein in microdiets for fish larvae is dependent on their amino acid profile. A sufficient supply of dietary amino acids is a prerequisite for high growth rates. Kanazawa *et al.* (1989) suggested that a MBD in which amino acid pattern approximated that of larval fish whole body protein produced good growth performance and survival of larval Japanese flounder. The suitability of dietary proteins as amino acid sources is thus an important issue in relation to larval nutrient requirements. In addition, amino acids seem to be a preferred energy substrate in larvae of the Atlantic halibut *Hippoglossus hippoglossus* (Rønnestad, Thorsen & Finn 1999) and the Atlantic cod *Gadus morhua* (Finn, Rønnestad, van der Meeren & Fyhn 2002). All MBDs contained 5% krill meal which has a favourable amino acid composition for some marine fish larvae and has been demonstrated to enhance food ingestion (Shimizu, Ibrahim, Tokoro & Shirakawa

1990), possibly due to its high free amino acid contents, which stimulates feeding behaviour in fish larvae (Kolkovski, Arieli & Tandler 1997). Previous studies on fish meal-based microdiets have reported limited success in milkfish *Chanos chanos* (Borlongan, Marte & Nocillado 2000) and winter flounder *Pseudopleuronectes americanus* (Ben Khemis, Audet, Fournier & De Lanouee 2003). Fish meal-based larval diets have also produced poor growth performance in larval cod *Gadus morhua* (Baskerville-Bridges & Kling 2000) and haddock *Melanogrammus aegle¢nus* (Blair, Castell, Neil, D'Abramo, Cahu, Harmon & Ogunmoye 2003) as poor growth observed in experiment 1 (MBD 1 and 2).

In experiment 2, dry body weight of larvae fed MBD 6 on 31 dph was significantly higher (1138 μg) than those fed the commercial microdiets (850 μg), indicating that MBD 6 could be used to reduce live *Artemia* feed cost for larval rearing of southern flounder. Compared to MBD 6, final body weights appeared to be slightly lower in larvae fed MBD 5, 7 and 8, but these differences were not significant. The favourable survival and growth of larvae fed MBD 6 could be due to the good digestibility, nutritional quality and acceptability of protein sources attributed to bioactive peptides, free amino acids, phospholipids and omega-3 fatty acids along with high levels of chemo-attractants such as alanine, glycine, taurine and betaine (Takaoka, Takii, Nakamura, Kumai & Takeda 1995; Tonheim, Nordgreen, Høgøy, Hamre & Rønnestad 2007).

Supplementation of feed attractants played an important role in acceptance of dry diets in fish larvae during the weaning period as well as in enhancing growth as reported for other species (Kolkovski *et al.* 1997; Kolkovski 2000). MBD 3, 4 and 6 contained squid meal which is rich in taurine and betaine (Sugiyama, Kousu, Hanabe & Okuda 1989). The chemo-attractant properties of squid meal were also confirmed for summer flounder *Paralichthys dentatus* (Lian, Lee & Bengtson 2008) and gilthead sea bream *Sparus aurata* (Kolkovski & Tandler 2000). The dietary inclusion of taurine produced a significant improvement in the growth of juvenile Japanese flounder (Kim, Takeuchi, Akimoto, Furuita, Yamamoto, Yokoyama & Murata 2005). In general, carnivorous species show a positive feeding response to alkaline and neutral substances, such as glycine, proline, valine, taurine and betaine (Takaoka *et al.* 1995).

In experiment 3, FBW of the southern flounder larvae fed only MBD 6 was significantly lower after 21 dph than the groups fed either live feed or co-fed live feed and MBD. Live feed organisms are thought to stimulate larval feeding and digestive system development by their movement and chemical cues (Kolkovski *et al.* 1997; Cahu & Zambonino-Infante 2001; Kolkovski 2001). Attempts have been made to rear larval and early juvenile fish and crustaceans with artificial microdiets, so-called artificial planktons (Kanazawa 1988; Jones 1998; Takeuchi, Wang, Furuita, Hirota, Ishida & Hayasawa 2003). Total replacement of live food with MBD has been accomplished in shrimp, but not in marine fish (Jones 1998; Cahu & Zambonino-Infante 2001). Therefore, it appears that weaning of 11 dph southern flounder larvae using only MBD may not be practical for mass larval production. However, in experiment 3, co-feeding MBD 6 and live prey produced better survival and growth compared to only MBD. Co-feeding of microdiets and live food generally produces an improvement in growth compared with feeding microdiet alone or live feed alone (Wang *et al.* 2009). However, an increase in the ingestion rate of MBD in the presence of live feed suggests that the zooplankters are stimulating appetite in the larvae resulting in improved MBD acceptance. Thus the present findings suggest that 50% *Artemia* could be replaced during rearing of southern flounder larvae using a high quality MBD.

Survival of southern flounder (68–84% at 35 dph) in experiment 1 was higher than in experiment 2 (24–31% at 34 dph) and experiment 3 (19.5–20.2% at 25 dph). This is because the larvae were fed the MBD starting 15 dph in experiment 1, 10 dph in experiment 2 and 11 dph in experiment 3. Lower survival for Experiments 2 and 3 suggest that 10–11 dph southern flounder larvae were not able to effectively digest MBD. The differences in survival of southern flounder larvae among the three feeding trials could also be due to different cohort, tanks' sizes and stocking densities. Although weaning the larvae from *Artemia* onto a MBD has been achieved in many marine finfish species, the early introduction of prepared diets as the sole replacement for live food has met with limited success (Kanazawa, Teshima, Inamori, Sumida & Iwashita 1982; Walford, Lim & Lam 1991; Dabrowski, Lee & Rinchard 2003). There is also a concern that the early addition of microdiets to the larval rearing tank can degrade water

quality and an increase in opportunistic bacterial communities (Olafsen 2001; D'Abramo 2002).

In general, the fatty acid composition of the fish whole body reflected the composition of their diets (Salhi & Bessonart 2013). The lipid sources of the commercial microdiet used in this study (Otohime) are unknown. However, the n-3 PUFA levels in the larvae fed the formulated MBD 6 were not significantly different from those fed the commercial microdiets (Otohime) after the feeding period. This was consistent with the similar growth and survival observed among these treatments. The high DHA content of flounder larvae reflects the importance of this fatty acid during larval development, which is known to have higher efficiency as an essential fatty acid than EPA (Watanabe & Kiron 1994). Growth, survival and fatty acid composition of larval whole bodies suggested that flounder larvae were able to utilize formulated zein bound microdiets, and the results are comparable with the commercially available microdiets.

The total dry matter leaching losses of the MBD 5, 6, 7, 8 and commercial diets after 10 min immersion in sea water were 27.8–30%. In this study (Experiments 2 and 3), the low survival of the larvae could be due to leaching losses of some essential soluble nutrients. Rapid rates of loss have been reported for free amino acids supplementing microdiets (Lopez-Alvarado & Kanazawa 1994; Kvåle, Yúfera, Nygård, Aursland, Harboe & Hamre 2006). Extensive leaching of nitrogenous compounds from microdiets based on intact fish protein sources demonstrated that leaching will also occur for water-soluble macromolecules. At the same time, some leaching of nutrients may be beneficial due to their roles as attractants (Kolkovski *et al.* 1997; Koven *et al.* 2001). A lower availability of the protein in formulated diets compared with live prey is considered to be an important reason for the low performance of marine fish larvae fed only formulated diets (Hamre, Næss, Espe, Holm & Lie 2001).

Conclusion

The southern flounder undergoes a true metamorphosis during which the bilaterally symmetrical larva transforms into an asymmetrical juvenile. Based on the results of this study, a suitable microbound diet could be formulated for southern flounder larvae from premetamorhic to late metamorphic stages (11–34 dph) using a combination of different protein sources and supplemental attractants. Formulated microdiets are also in demand for scientific purposes, to increase the possibilities to manipulate and control the dietary nutritional composition in feeding trials with southern flounder larvae. Good growth and survival of southern flounder larvae (10–35 dph) were achieved with co-feeding of MBD 6 containing fishmeal, squid meal, krill meal, herring meal and attractants as the major nitrogen sources, suggesting that larvae can assimilate these dietary proteins for growth.

Acknowledgement

This research was supported by the North Carolina Sea Grant (grant number 08-AQ-09) and MARBIONC (Marine Biotechnology in North Carolina) Center for Marine Science, University of North Carolina Wilmington, USA.

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