Influence of the timing of weaning on growth and survival of juvenile winter flounder (*Pseudopleuronectes americanus*)

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Abstract

Metamorphosis is a critical developmental stage that presents particular challenges in fish aquaculture. The sharp increase in mortality that accompanies this transformation has often been attributed to nutritional deficiencies. Providing live feed (rotifers [*Brachionus plicatilis* (Müller, 1786)], *Artemia* [*Artemia salina* (L., 1758)]) during the larval stages is costly and labour-intensive, which explains why much effort has been put on early weaning. However, previous observations in winter flounder (*Pseudopleuronectes americanus* (Walbaum, 1792)) indicate that juveniles weaned after settlement had better survival than those weaned at the larval stage. In this study, we tested whether late weaning (at settlement [W0], and groups maintained on co-feeding for one [W1], two [W2], or three [W3] months following settlement) could improve juvenile survival and lipid composition. Our results demonstrated that maintaining co-feeding beyond the larval stage was essential for post-settlement survival. Juveniles co-fed until 90 days after settlement were 32.5% heavier. Analyses of fatty acids trophic markers suggested that juveniles preferentially fed on enriched rotifers rather than inert food. No pigmentation or fin erosion problems were observed in any of the weaning treatments, which indicates good rearing conditions.

Résumé

La métamorphose est une étape critique du développement qui présente des défis particuliers en aquaculture. La forte hausse de mortalité associée à cette étape a souvent été attribuée à des carences nutritionnelles. Fournir de la nourriture vivante (rotifères [*Brachionus plicatilis* (Müller, 1786)], *Artemia* [*Artemia salina* (L., 1758)]) au cours des premiers stades de vie est

coûteux et exige beaucoup d'efforts ce qui explique l'intérêt pour un sevrage rapide. Cependant, des observations précédentes chez les juvéniles de plie rouge (*Pseudopleuronectes americanus* (Walbaum, 1792)) indiquent que des juvéniles sevrés au moment du passage à l'habitat benthique ont une meilleure survie que ceux sevrés au stade larvaire. Dans cette étude, nous avons testé si un sevrage tardif (au moment du passage à la phase benthique [W0]) et chez des groupes maintenus en alimentation mixte pour un [W1], deux [W2], ou trois [W3] mois suivant le début de la phase benthique) pouvait accroître la survie et améliorer les teneurs en lipides. Nos résultats indiquent que le maintien de l'alimentation mixte au-delà du stade larvaire est essentiel pour améliorer la survie suite au passage en phase benthique. Les juvéniles nourris sur un régime mixte jusqu'à 90 jours suivant le passage en phase benthique avaient une masse 32.5 % plus élevée. L'analyse de marqueurs trophiques parmi les acides gras suggère que les juvéniles se nourrissent préférentiellement de rotifères. L'absence de problèmes de pigmentation ou d'érosion de nageoire au cours des différents traitements indique la présence de conditions d'élevage adéquates.

Keywords: Winter flounder, *Pseudopleuronectes americanus*, weaning, live prey, lipid classes, fatty acids, aquaculture

Introduction

Winter flounder (*Pseudopleuronectes americanus* (Walbaum, 1792)) is a common flatfish that inhabits coastal waters of the northwest Atlantic, from Georgia (33˚N) to Newfoundland and southern Labrador (53˚N; Buckley 1989). It has been identified as a promising species for aquaculture in Eastern Canada (Litvak 1999) because it is hardy; it is euryhaline (McCracken 1963), eurythermal (Pearcy 1962; Duman and Devries 1974) and produces antifreeze proteins allowing it to survive below the freezing point of water (Duman and Devries 1974; Fletcher 1977). It can easily be kept in captivity, and Litvak (1999) indicated that adults grown in cages were bigger than wild stocks. Based on previous work done on the larval stage (e.g., Audet and Tremblay 2011), this species is a good model to improve feeding procedures during the transition period between the larval and juvenile stages.

Winter flounder have been grown to adults from eggs under hatchery conditions since the 1990's. Brood stock management (Plante et al. 2003) and larval rearing have been achieved (Ben Khemis et al. 2000; 2003; Mercier et al. 2004), and newly metamorphosed juvenile rearing conditions have been explored (Lee and Litvak 1996; de Montgolfier et al. 2005). Nevertheless, specific knowledge on stock enhancement, breeding, and both larval and juvenile rearing is lacking to make this production possible for the industry (Audet and Tremblay 2011). As is the case for most marine fishes (Cahu and Zambonino-Infante 2001), information on nutritional requirements that would allow the development of mass production systems is currently lacking.

Flounder undergo an important metamorphosis between the larval and juvenile stages. Changes in behaviour and ecology—and more importantly in morphology and physiology—in this critical developmental stage cause drastic energy depletion and a decrease in survival

(Fraboulet et al. 2010). At this stage, it is crucial to provide quality feed to ensure the success of metamorphosis while also limiting the cost of the feed. Live prey are the main food source of cultured marine fish larvae. However, the production and use of live feed is expensive. Live feed are currently used because artificial food alone cannot ensure growth and survival, especially in the very early stages of development (Conceição et al. 2010).

In winter flounder, enriched rotifers (*Brachionus plicatilis* (Müller, 1786)) are required from the first feeding, followed by the use of *Artemia salina* (L., 1758) when larvae increase in size (~ 5.5 mm; Ben Khemis et al. 2003). Live prey such as rotifers and *Artemia* are naturally poor in nutrients and need to be enriched, especially in essential unsaturated fatty acids (EUFA) such as docosahexaenoic acid (DHA; 22:6n-3), eicosapentaenoic acid (EPA; 20:5n-3), and arachidonic acid (AA; 20:4n-6) (Wanatabe and Kiron 1994; Sargent et al. 1999; Conceição et al. 2010). Indeed, the biosynthesis capacity of marine fishes for EUFA is very limited (Sargent et al. 1999; Glencross 2009), so these must be provided in the diet. Co-feeding a mixed diet of live prey and commercial food to marine fish larvae has been shown to sustain growth and survival while reducing production costs (Roselund et al. 1997; Canãvate and Fernandez-Díaz 1999; Koven et al. 2001). Weaning to commercial micropellets must be achieved to optimize juvenile production in hatcheries. Very early in their development, winter flounder are equipped with the enzymes needed to digest lipids (Murray et al. 2003) and proteins (Mercier et al. 2004; Murray et al. 2004), suggesting that they are able to digest inert food in their first life stages. Ben Khemis et al. (2003) showed that feeding larvae a mixed regime resulted in no reduction in growth rate until metamorphosis. These authors also showed that a rapid weaning (four days) starting at 5.5 mm standard length significantly lowered growth, while no effect was found on growth or onset of metamorphosis when weaning started at 6.6 mm. Butts et al. (2015) also found that winter flounder were most

receptive to an artificial diet between 5.5 and 6.23 mm. The optimal timing of weaning for winter flounder needs to be determined since it plays an important role in rearing success (Conceição et al. 2010),

This study explores the possibility of co-feeding winter flounder larvae with enriched rotifers and a commercial microdiet with different post-settlement weaning onset times. We hypothesized that maintaining a long-term co-feeding regime after settlement will reduce the mortality observed during metamorphosis, and that this reduced mortality is related to a higher content of essential fatty acids in juveniles.

Material and Methods

Experimental design and rearing conditions

Larvae were reared as described by Ben Khemis et al. (2003) except that enriched rotifers were the only live prey used during the experiment (no *Artemia*). At the end of the larval phase, the bottoms of the cylindro-conical larval rearing tanks were gently siphoned every two days to collect newly settled juveniles that were then transferred to rectangular flat-bottomed juvenile rearing tanks (35.5 × 65 × 6.5 cm). Water inflow was at one side (of the longer axis) and the drain was on the opposite side. In each tank, water flow was adjusted to 1.4 L min^{-1}. Individuals with a flattened body and completed eye migration were identified as juveniles (Chambers and Leggett 1987). The bottoms of the flat tanks were covered with 250 mL of sand. Juveniles were stocked at an initial density of 400 juveniles per tank.

Juveniles were fed a mixed diet of enriched rotifers and commercial inert food (Gemma Wean Diamond, Skretting, France) until weaning. Different weaning times were tested: at settlement (W0), and one (W1) and two (W2) months after settlement; a fourth group (W3) was

maintained on co-feeding until the end of experiments (90 days following settlement). Weaning was accomplished within three days with a progressive reduction of rotifers (by 1/3 per day) until feeding with only commercial inert food. Four juvenile rearing tanks were used for each treatment.

Each juvenile tank under the mixed diet treatment received 30 000 enriched rotifers twice a day. Rotifers were enriched with a live microalga concentrate (5×10^9 cells per 10^6 rotifers) (*Nannochloropsis* sp. (Hibberd, 1981)*, Isochrysis galbana* (Parke)*, Pavlova lutheri* (Green, 1975)*,* 1:1:1 v:v:v; Nutrocean, Rimouski, QC, Canada) and enriched daily with Selco (1 g 10^6 rotifers) (INVE Aquaculture nutrition, Gransville, UT, USA). A liquid concentrate (Nutrocean) of live microalgae (0.7 cells L^{-1}) was also added to juvenile rearing tanks after the addition of rotifers. Each juvenile rearing tank received a daily ration of 0.2 g of commercial food (Gemma Wean Diamond, INVE) given in nine meals. At each dry food meal, the water's surface was gently agitated so that food particles would sink. The size of the commercial food was adjusted with juvenile growth: from day 0 to day 30 after settlement, we used the 0.3 mm pellets; from day 30 to day 60, juveniles received a 50:50 mix of 0.3 and 0.5 mm pellets; and after 60 days, they were given the 0.5 mm pellets only. Seawater flow was stopped from 09h00 to 12h00 and from 13h00 to 15h30 to avoid loss of rotifers and food by water renewal during feeding.

Sea water was pumped from the St. Lawrence estuary and sand filtered (50 µm) prior to entering the rearing tanks at 2 L min^{-1}. Rearing was done under natural conditions of photoperiod (400 lx), salinity (28–30), and temperature (from 12°C in summer to 6°C at the end of the experiment). Each tank was cleaned daily to remove excess food and dead

individuals using sterile plastic Pasteur pipets. A thorough cleaning was done once a month during which the remaining fish were counted.

Sampling

Juvenile samplings were done 0, 15, 30, 45, 60, 75, and 90 days after settlement for each weaning treatment for survival measurements. Fish were imaged using a digital camera (Evolution^{MD} VF, Media Cybernetics) coupled to a binocular microscope (Olympus SZ61). On day 0, six juveniles per larval rearing tank were imaged. For all other sampling days, 10 juveniles were imaged per replicate tank (160 juveniles per sampling day). On days 45 and 75, juveniles were returned to their respective rearing tanks after being imaged. For sampling days 15, 30, 60, and 90, six of the 10 sampled juveniles were weighed on an analytical balance, frozen, and stored at -80ºC for lipid analyses.

Rotifers were sampled every three weeks from 5 July until the end of the experiment. Samplings were done in the morning before addition of microalgae and enrichment. At each sampling, volumes of ~ 20 000 rotifers were filtered and dried to determine dry weight (70°C for 24h) or stored at -80ºC until lipid analysis. Three samples of 0.2 g of commercial Gemma Wean Diamond feed were also stored at -80ºC for lipid analysis.

Standard length and body width were measured for each imaged juvenile. The levels of pigmentation and fin erosion were evaluated qualitatively according to de Montgolfier et al. (2005). Measurements were done using Image Pro-Plus 5.0 (Media Cybernetics, Silver Spring, MD, USA). Mean values of measurements for each tank were used for statistical analysis.

Lipids were extracted in dichloromethane–methanol according to Parrish (1999) using a modified Folch procedure (Folch et al. 1957). Extracts were spotted on silica gel-coated chromarods (SIII, Shell USA). Lipid classes were determined according to Parrish (1987); they were separated into triacylglycerols (TAG), free sterols (ST), phospholipids (PL), acetone-mobile polar lipids (AMPL), free fatty acids (FFA), wax esters (WE), ketones (KET), and free aliphatic alcohol (ALC) by thin-layer chromatography using flame ionization detection (TLC/FID) with an Iatroscan MK-6 (Shell USA, Fredericksburg, VA, USA). Lipid classes were evaluated in mg g^{-1} of wet tissue, summed, and expressed as a percentage of total lipids. Fatty acids were analyzed on these lipids extracts, but only for inert commercial foods, enriched rotifers, and juveniles sampled at 90 days for the W2 and W3 weaning treatments. Neutral and polar lipids from extracts were separated by silica columns and eluted by dichloromethane:methanol and methanol, respectively (Marty et al. 1992). Methylation was done by addition of sulfuric acid and heating at 100ºC for one hour. To measure neutral lipids, an additional elution was done with hexane and diethyl ether. Fatty acid methyl esters (FAME) were analyzed on a Polaris Q ion trap coupled to a Trace GC (ThermoFinnigan) equipped with a VB-5® mass spec grade fused silica capillary column (30 m × 0.25 mm i.d; ValcoBond®, USA) with high-purity helium as the carrier gas. Detection and quantification of FAME were conducted in full-scan mode with positive ions, with a scan range of 60–650 amu. External calibration was used with appropriate dilution in hexane:dichloromethane of Supelco 37 component FAME Mix standard solution to obtain five-point calibration plots, from 6.25 to 100 µg ml^{-1}. Xcalibur® software (Version1.3) was used to control the system and for data acquisition and processing.

Statistical analyses

STATISTICA 6.0 software (Statsoft© 2001) with a level of significance $\alpha = 0.05$ was used. Normality and homoscedasticity were verified respectively with the Kolmogorov-Smirnov distribution test and the Brown-Forsythe test. If necessary, percentage data (survival, lipid classes) and lipid ratios (TAG:ST, EPA:DHA, DHA:AA, EPA:AA) were arcsine–square-root transformed prior to analysis (Sokal and Rohlf 1995). The averages from each replicate tank were used as the statistical unit ($n = 4$ per treatment per sampling day). At each sampling time (0, 15, 30, 45, 60, 75, and 90 days after settlement), one-way ANOVAs were used to determine weaning effect (W0, W1, W2, W3) on survival, standard length, width, weight, concentration of total lipids, and proportions of lipid classes. Two-way ANOVAs could not be used because some treatments were eliminated along the way due to high mortality rates. If significant differences were detected, Tukey HSD multiple comparisons tests were applied. To explore differences in fatty acid compositions between inert food, rotifers, and the neutral and polar lipid fractions of juveniles sampled 90 days after settlement for the W2 and W3 treatments, a permutational multivariate analysis of variance (PERMANOVA with 9999 permutations) was applied using PRIMER 6.1.12 with PERMANOVA+ 1.0.2 based on Bray-Curtis matrix. A posteriori comparisons were done using a PERMANOVA pairwise test while non-metric multi-dimensional scaling (n- MDS) and SIMPER analyses were performed to examine the similarity between profiles.

Results

The different weaning times had a significant effect on juvenile survival (Fig. 1). Very high mortality occurred in the month following settlement in juveniles weaned at settlement (W0), and none of the W0 juveniles survived until day 60. At 30 days, the W1, W2, and W3 treatments—where weaning had not yet begun—showed similar survival rates (20–30%). A mean survival of 2.5% was observed for W1 juveniles one month after being weaned (day 60)

compared to around 15% for W2 and W3. Three months after settlement, only 0.4% of W1 juveniles remained, and survival for treatments W2 and W3 was around 7%. Thus at day 90, W2 (30 days after weaning) showed a survival rate similar to W3 (no weaning). At settlement, juveniles had a mean standard length of 6.64 ± 0.26 mm and a mean width of 1.98 ± 0.13 mm. A significant difference in standard length was only observed on day 45 among W1, W2, and W3 juveniles (Table 1), with the W1 juveniles being 8.9% smaller than W3 juveniles, while the average length of W2 juveniles was not statistically different from either W1 or W3 juveniles. No significant difference was observed in terms of width except on day 15, when W3 juveniles were 14% larger than W0 juveniles (Table 1). Increases in mass were similar among weaning treatments except on day 90, when the weight of W3 juveniles was 30% greater (Table 1).

The main lipid classes in enriched rotifers were phospholipids (63%) and triacylglycerols (25%) while those of the inert commercial food were ketones (73%) and phospholipids (12%) (Table 2). Juveniles did not exhibit any significant differences between sampling time and weaning treatment in terms of lipid concentration or lipid class composition until day 90. On day 90, W3 juveniles had 12, 4, and 2.2 times more ketones, triacylglycerols, and acetone-mobile polar lipids, respectively, and 35.1% less phospholipids compared to the W2 juveniles; all these differences were significant (Table 2). Free fatty acids, wax esters, and aliphatic alcohol are not reported because values were less than 1% of total lipids.

We observed significant differences in the fatty acid (FA) composition of enriched rotifers, the commercial diet, and the neutral and polar fraction of W2 and W3 juveniles on day 90 (one-way PERMANOVA, pseudo-$F_{(5,22)} = 36.4$, $p < 0.001$), and this was also shown by n-MDS analysis (Fig. 2). Significant differences were observed between each pair-wise

comparison ($p < 0.016$). SIMPER analyses indicated that the fatty acid profiles of inert commercial food showed 39% dissimilarity with those of rotifers. The difference was mainly related to the 16:0 and 16:1n7 fatty acids (Table 3). The fatty acid profiles of inert commercial food and rotifers showed 39% and 28.9% dissimilarities with the neutral lipid fraction of the W2 weaned juveniles that was mainly related to 16:0 and 20:3n3 for inert food and to 16:1n7, 22:6n3, and 20:5n3 for rotifers (Table 3). Similar results were observed for the neutral lipid fraction of W3 juveniles, with 43.7% dissimilarity with inert commercial foods and 21.2% with rotifers; the same fatty acids contributed to these differences (Table 3). These results were confirmed by the n-MDS analysis, which showed that FA profiles from rotifers more closely resembled those of the W2 and W3 neutral lipid fractions than those of the inert commercial foods. The neutral lipid fractions of W2 and W3 juveniles showed significant differences, but only 20% of the dissimilarity was explained by 16:1n7 and 22:6n3. The neutral and polar lipid fractions of W2 showed 26.1% dissimilarity that was explained by 18:0, 22:6n3, and 16:0, while the neutral and polar lipid fractions of W3 juveniles showed 27.1% dissimilarity that was explained by 16:1n7, 16:0, and 18:0.

Discussion

Our results demonstrate the possibility of using enriched rotifers and a commercial diet to co-feed winter flounder juveniles. The use of fatty acid analyses to identify trophic markers clearly showed that winter flounder juveniles preferentially fed on enriched rotifers until 90 days after settlement. However, mortalities of over 70% observed 30 days after settlement in all treatment suggest that there are nutritional deficiencies in the larval or post-larval feed. This species undergoes an important metamorphosis involving a 90° rotation in body position, the development of asymmetrical pigmentation, and the migration of one eye towards the other on the upper side of the fish (Fuiman 1997). These metamorphic processes exact high

energy costs (Gwack et al. 2003), and post-larvae mobilize most of their triacylglycerol reserves shortly after settlement (Fraboulet et al. 2010; 2011). Thus, individuals that do not accumulate enough energetic reserves during larval development do not survive metamorphosis (Geffen et al. 2007).

A previous genomic study suggested that the first 30 days after settlement correspond to a metamorphosis maturation process where the larva stays settled without growth, and where an increase in aerobic capacity occurs with a switch from the pentose-phosphate pathway to lipid metabolism as an energy source for ATP synthesis to sustain juvenile growth (Vagner et al. 2013). Here, the low level (< 5%) of accumulated TAG (the major energy storage form in marine fishes; Lochman et al. 1995) by 30 days after settlement in the surviving juveniles could suggest a lack of sufficient energy reserves necessary to support metamorphosis. It is also possible that the cost of hunting live prey during metamorphosis could be decreased by the use of larger prey such as *Artemia*, and that this would increase survival 30 days after settlement. However, survival was good in the co-feeding treatments between 30 and 90 days after settlement (less than 30% mortality), suggesting that the use of small rotifers as live prey is viable. Finally, we confirm the hypothesis that a long-term co-feeding regime increases survival through metamorphosis, suggesting that there were nutritional deficiencies with the use of artificial food only.

Weaning effect

This study demonstrates that the timing of weaning not only influences growth and survival of newly settled winter flounder juveniles, but also that maintaining co-feeding beyond the larval stage is essential for post-settlement survival. It is uncommon to use enriched rotifers as the only live prey: a succession of different prey increasing in size is usually used for fish growth

(Guillaume et al. 2001; Lucas and Southgate 2003). Using rotifers only makes larval and juvenile culture simpler, and we assumed that the ingestion of commercial food could be stimulated by the use of smaller live prey. The 0.063 mm day^{-1} growth of surviving juveniles (W2 and W3) was similar to previous reports under laboratory conditions (Chambers et al. 1988), and the 0.27 mm day^{-1} (W2) and 0.40 mm day^{-1} (W3) weight gains were superior.

The lipid results indicate that juveniles until 90 days assimilated very little of the inert commercial food. This commercial food was characterized by high levels of ketones and fatty acid profiles that were different from the neutral lipid fraction of juveniles after 60 and 90 days of co-feeding. Fatty acid incorporation into neutral lipids is less regulated than fatty acid incorporation into polar lipids, thus fatty acids in the neutral lipid fraction more closely reflect dietary concentrations (Delaunay et al. 1993; Skalli and Robin 2004). Since the neutral lipids in the fatty acid profiles of juveniles are more similar to those of rotifers than to those of inert food, these results suggest that juveniles seem to preferentially use rotifers.

Mono- and polyunsaturated fatty acids (MUFA and PUFA), especially those contained in neutral fatty acids, are strongly influenced by the food source (Blair et al. 2003). The two fatty acids characteristic of the commercial inert food (high levels of 22:1n-9 and 20:3n-3) were lower in the neutral lipid fractions of W2 and W3 juveniles, suggesting that juveniles have difficulty assimilating this inert food. However, total MUFA content in juveniles clearly indicated that rotifers were still part of the diet of co-fed juveniles. Rotifers have more than twice as much MUFA compared to the commercial food, and this is clearly reflected in W3 juveniles, whose proportion was 1.5-fold higher in neutral lipids compared to W2 juveniles. However, the high level of 22:6n3 (DHA) in the neutral lipids of juveniles was more similar to the level observed in inert food, suggesting that fish still assimilated some of the feed's

fatty acids. DHA is an important essential fatty acid; it is highly regulated and has a high retention level in the polar lipid fraction, as observed in our results on winter flounder juveniles. The inert food is also characterized by the presence of ketones as the main lipid class (73%). In juveniles, a high level of ketones was observed only at day 90 for juveniles that had been co-fed for 90 days (W3). The observed ketone proportion (20% of total lipids) in the juveniles represents a much higher value than what has been previously found for winter flounder larvae (maximum of 5% in pelagic larvae; Vagner et al. 2014) and juveniles (maximum of 10% from settlement to 45 days post-settlement; Fraboulet et al. 2010; Vagner et al. 2014), supporting the possible use of the commercial inert food by W3 juveniles. The function of ketones in fish metabolism is still unknown (Alkanani et al. 2005), but it could provide an alternative lipid-based energy source (Ballantyne 2004). Elasmobranch fishes use ketone bodies as an energy source (Zammit and Newsholme 1979), but marine teleost fishes generally show no enhanced capacity for ketone body metabolism, even during food deprivation (Zammit and Newsholme 1979; Segner et al. 1997).

Weaning newly settled juveniles at the same time as they undergo metamorphosis is clearly not ideal, even though they should be at a period in their life-cycle that corresponds to shifts in feeding regime and behaviour (transition from pelagic to benthic habitat and prey). Ben Khemis et al. (2003) suggested that winter flounder can be weaned at a standard length of 6.6 mm, although their experiment only covered the larval period and ended at settlement, while Lee and Litvak (1996) focused on weaning well after metamorphosis at a standard length of 12.5 mm. It is assumed that the presence of live prey in the water column increases the feeding rate. Kolkvoski et al. (1997) showed that the feeding response on commercial food in sea bream larvae increased in the presence of the chemical and visual stimuli of live prey. Such positive effects of co-feeding were also observed in other flatfish species

(Roselund et al. 1997; Chang et al. 2006). Similar results were obtained in a weaning experiment on Senegalese sole: weaned fish did not show growth difference compared to those fed live prey even though they exhibited a higher mortality rate (Cañavate and Fernàndez-Dìaz 1999). These authors suggested that a selection occurred for individuals that were better able to assimilate the commercial food.

Nutritional needs

Successful weaning of marine fishes depends on adequate feeding behaviour and a diet that meets the species' specific nutritional requirements (Cahu and Zambonino Infante 2001). Most studies on nutritional requirements of marine fishes have highlighted the important role of lipids (e.g., Sargent et al. 1999; Trushenski et al. 2006; Glencross 2009). Lipids represent the energy source for larval growth, and some polyunsaturated fatty acids are essential (AA, EPA, and DHA) for cell membrane function and integrity. EPA and DHA are incorporated into membrane phospholipids and are involved maintaining the structural and functional integrity of biological membranes (Hulbert and Else 1999), while AA and EPA are precursors of eicosanoids, a group of highly biologically active hormones (Howard and Stanley 1999). These fatty acids have been demonstrated to be essential in marine fishes. DHA has been observed in very high concentrations in neural and visual membranes, while EPA and AA increased stress tolerance in marine fishes (Sargent et al. 1999; Bell and Sargent 2003; Glencross 2009). Here, the proportions of EPA and DHA were higher in juveniles than in rotifers, indicating selective retention in polar lipids. Thus, our results suggest that rotifers were not sufficiently enriched in EPA and DHA, and that the EPA content in inert food was also insufficient. The presence of saturated fatty acids (SFA) in feed is also important because energy is more efficiently released via β-oxidation of SFAs than of PUFAs (Langdon and Waldock 1981). Thus, the higher level of SFA in inert food could be beneficial for juvenile

growth if it was preferentially used by winter flounder juveniles. Levels of amino acids, vitamins, and minerals in the feed, which were not characterized in this study, are other essential dietary elements necessary for survival and growth of marine fishes (Lucas and Southgate 2003).

Long-term co-feeding allowed W3 juveniles to get heavier. The increased mass could be related to the high level of phospholipids in rotifers compared to inert food (63% vs 12%, respectively). In sea bass, the phospholipase A2 content suggests that young juveniles digest phospholipids more efficiently than triacylglycerols (Cahu et al. 2003). Thus, the use of rotifers to feed young juveniles could facilitate lipid digestion. Furthermore, fish have a limited capacity to produce phospholipids *de novo* (Geurden et al. 1995; Fontagne et al. 1998).

It should also be noted that a very low number of juveniles showed fin erosion and that pigmentation developed normally. Larval diet is thought to play an important role in the development of pigmentation in marine fishes (Estèvez et al. 1999; Copeman et al. 2002; Bell et al. 2003; Lund et al. 2007). AA is considered as the preferred precursor for eisocanoid production; however, an excess of eisocanoids can also be associated with pigmentation failure (Estèvez et al. 1999; Copeman et al. 2002).

In conclusion, long-term co-feeding enhanced juvenile survival and growth. Keeping live prey in the diet seemed to stimulate feeding on both sources, as indicated by lipid composition analyses. Nevertheless, high mortality was observed mainly during the first 30 days following settlement. Further studies comparing larval feeding with natural prey as well as surveys on

the nutritional state of wild larvae are in progress and may help find ways to better fulfill nutritional needs and improve survival rates in production.

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Table 1. Standard length, width, and wet weight of winter flounder (*Pseudopleuronectes americanus*) juveniles for the different weaning periods (at settlement [W0]; one [W1], two [W2], and three [W3] months after settlement). Results are expressed as mean ± S.D. *F* and *P* are provided for each sampling day and each variable; significant values are in bold. Different letters indicate significant differences among treatments of the same sampling day.

Days after Settlement			Standard length (mm)	Width (mm)	Wet weight (mg)
0	*F*, *P*		0.91, 0.46	0.64, 0.60	
		W0	6.8 ± 0.18	2.1 ± 0.10	
		W1	6.6 ± 0.20	2.0 ± 0.09	
		W2	6.6 ± 0.23	1.9 ± 0.12	
		W3	6.5 ± 0.33	2.0 ± 0.21	
15	*F*, *P*		1.86, 0.19	3.67, **0.04**	1.39, 0.29
		W0	6.7 ± 0.29	2.1 ± 0.15^{a}	3.6 ± 0.65
		W1	7.0 ± 0.22	2.3 ± 0.09^{ab}	4.5 ± 0.29
		W2	7.0 ± 0.15	2.3 ± 0.08^{ab}	4.2 ± 0.64
		W3	7.2 ± 0.34	2.4 ± 0.17^{b}	4.9 ± 1.61
30	*F*, *P*		1.06, 0.41	2.14, 0.15	0.64, 0.60
		W0	7.7 ± 0.22	2.5 ± 0.13	6.7 ± 1.11
		W1	8.1 ± 0.32	2.8 ± 0.11	8.2 ± 3.59
		W2	8.0 ± 0.49	2.7 ± 0.31	8.2 ± 1.16
		W3	8.0 ± 0.17	2.9 ± 0.05	8.9 ± 1.18
45	*F*, *P*		6.27, **0.02**	3.58, 0.07	
		W0			
		W1	8.2 ± 0.10^{a}	2.9 ± 0.06	
		W2	8.8 ± 0.31^{ab}	3.1 ± 0.17	
		W3	9.0 ± 0.43^{b}	3.2 ± 0.20	
60	*F*, *P*		0.32, 0.73	0.36, 0.71	0.58, 0.58
		W0			
		W1	10.4 ± 0.31	3.8 ± 0.22	16.6 ± 2.53
		W2	10.2 ± 0.74	3.7 ± 0.35	18.6 ± 5.61
		W3	10.6 ± 0.76	3.9 ± 0.34	20.1 ± 5.00
75	*F*, *P*		1.87, 0.22	1.11, 0.38	
		W0			
		W1	11.5 ± 0.03	4.3 ± 0.03	
		W2	10.9 ± 0.47	3.9 ± 0.25	
		W3	11.0 ± 0.36	4.0 ± 0.30	

90	*F*, *P*		1.42, 0.28	1.89, 0.22	15.08, **<0.01**
		W0			
		W1			
		W2	12.3 ± 0.79	4.4 ± 0.33	24.7 ± 3.50[a]
		W3	12.5 ± 0.32	4.7 ± 0.10	34.6 ± 3.66[b]

Table 2. Total lipids (mg g_{ww}^{-1}) and lipid class proportions (% of total lipids) in inert commercial food, enriched rotifers (*Brachionus plicatilis*), and winter flounder (*Pseudopleuronectes americanus*) juveniles for the different weaning periods (at settlement [W0]; one [W1], two [W2], and three [W3] months after settlement). Juveniles did not exhibit any difference between sampling time and weaning treatment in terms of lipid concentration or lipid class composition until day 90. Mean ± S.D. Bold indicates significant differences at 90 days after settlement between W2 and W3.

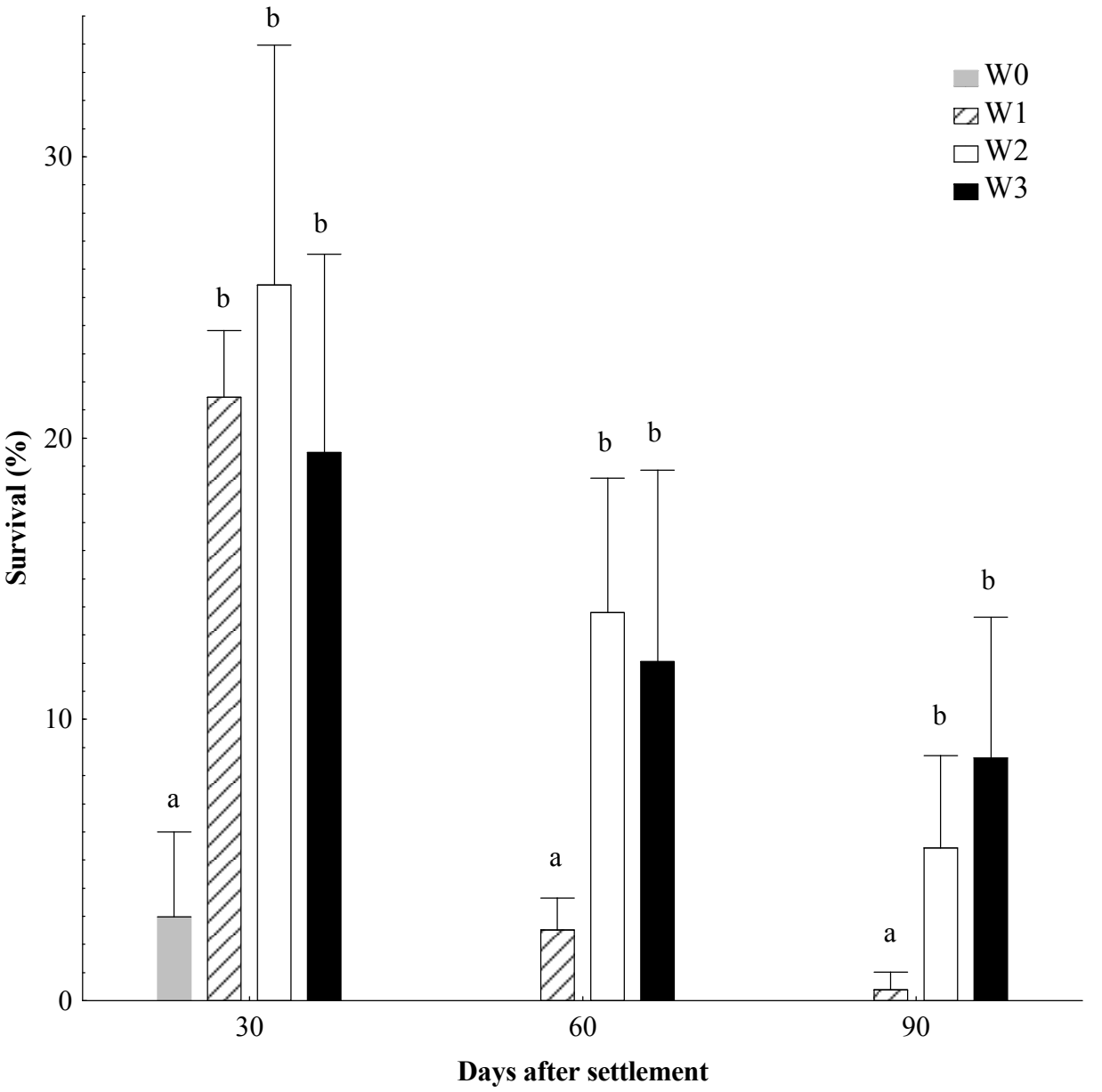
	Inert food	Rotifers	0 to 60 days W0 to W3	90 days W2	90 days W3
Total lipids (mg g_{ww}^{-1})	132.8 ± 13.8	72.9 ± 24.6	16.4 ± 8.7	12.2 ± 0.6	13.6 ± 5.3
% total lipids					
KET	73.3 ± 1.3	3.1 ± 3.2	5.8 ± 6.1	**1.7 ± 1.5**	**20.9 ± 15.6**
TAG	6.3 ± 0.7	25.1 ± 8.8	4.3 ± 4.8	**2.8 ± 2.1**	**11.1 ± 5.3**
ST	4.2 ± 0.3	1.3 ± 0.7	8.2 ± 4.2	14.4 ± 3.4	9.6 ± 5.7
AMPL	4.3 ± 0.3	7.6 ± 7.5	5.3 ± 3.5	**3.7 ± 1.9**	**8.3 ± 1.9**
PL	11.9 ± 1.1	63.1 ± 14.1	76.4 ± 11.8	**77.4 ± 3.6**	**50.2 ± 16.8**
TAG/ST	1.5 ± 0.3	32.9 ± 13.5	0.7 ± 0.9	0.2 ± 0.2	3.4 ± 5.1

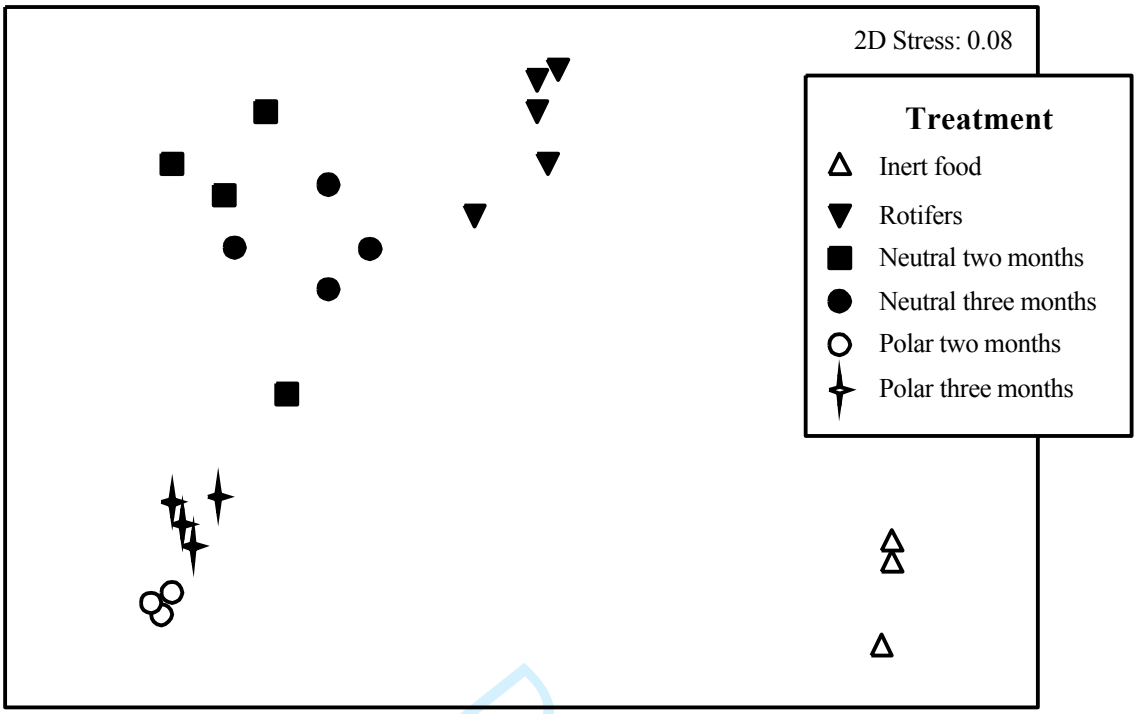
Table 3 Proportions of the different fatty acids (% of total neutral fatty acids) in food sources (inert food and rotifers) and the neutral and polar lipid fractions of winter flounder (*Pseudopleuronectes americanus*) juveniles on day 90 weaned two (W2) or three (W3) months after settlement. Mean ± S.D.

	Inert food	Rotifers	Neutral W2	Neutral W3	Polar W2	Polar W3
14:0	0.8 ± 0.3	3.3 ± 1.0	3.5 ± 0.2	3.9 ± 0.7	1.2 ± 0.1	1.6 ± 0.1
15:0	0.8 ± 0.1	0.9 ± 0.4	0.9 ± 0.4	0.7 ± 0.1	0.4 ± 0.1	0.2 ± 0.2
16:0	32.9 ± 0.8	13.5 ± 6.2	13.8 ± 1.0	12.7 ± 0.7	19.0 ± 1.4	19.9 ± 0.7
17:0	0.8 ± 0.1	0.6 ± 0.1	1.2 ± 0.5	0.7 ± 0.2	0.6 ± 0.1	0.6 ± 0.1
18:0	7.8 ± 0.3	5.6 ± 0.8	5.4 ± 0.1	5.2 ± 0.9	10.8 ± 0.4	10.6 ± 0.4
20:0	0.5 ± 0.1	0.6 ± 0.1	1.4 ± 0.8	0.6 ± 0.3	0.3 ± 0.1	0.2 ± 0.1
21:0	0.1 ± 0.1	0.5 ± 0.1	1.2 ± 0.8	0.5 ± 0.3	0.2 ± 0.1	0.1 ± 0.1
22:0	0.3 ± 0.1	0. 3 ± 0.1	1.7 ± 1.1	0.7 ± 0.3	0.3 ± 0.1	0.2 ± 0.1
24:0	0.4 ± 0.1	0.7 ± 0.1	1.1 ± 0.9	0.6 ± 0.3	0.3 ± 0.1	0.3 ± 0.1
ΣSFA	44.5 ± 0.8	26.1 ± 6.1	30.3 ± 3.8	25.7 ± 0.9	33.1 ± 1.4	33.6 ± 1.1
16:1n7	7.8 ± 0.7	19.1 ± 1.0	8.2 ± 1.4	16.2 ± 3.4	3.5 ± 0.2	6.3 ± 0.7
18:1n7	0.2 ± 0.1	6.1 ± 0.6	5.3 ± 0.7	7.8 ± 1.1	5.0 ± 0.2	6.9 ± 1.2
18:1n9	1.6 ± 0.4	8.8 ± 0.8	7.0 ± 1.6	10.6 ± 1.2	7.3 ± 0.3	7.3 ± 0.6
20:1	1.1 ± 0.2	4.8 ± 0.2	2.8 ± 0.4	3.6 ± 0.5	2.4 ± 0.1	2.7 ± 0.1
22:1n9	6.1 ± 0.8	3.3 ± 0.2	2.7 ± 0.2	1.8 ± 0.5	0.6 ± 0.1	0.6 ± 0.1
24:1n7	1.8 ± 0.1	1.5 ± 0.1	1.4 ± 1.1	0.7 ± 0.5	2.6 ± 0.4	1.9 ± 0.3
ΣMUFA	18.4 ± 1.1	40.3 ± 1.8	27.4 ± 2.6	40.7 ± 5.9	21.4 ± 0.3	25.7 ± 0.4
18:2n6	4.8 ± 1.2	8.8 ± 0.3	3.2 ± 0.2	5.1 ± 1.2	6.1 ± 0.5	7.1 ± 0. 6
20:2	0.4 ± 0.1	1.2 ± 0.1	1.9 ± 0.4	1.1 ± 0.2	0.7 ± 0.1	0.7 ± 0.1
22:2	4.2 ± 1.1	3.5 ± 0.2	1.1 ± 0.6	0.4 ± 0.2	0.2 ± 0.1	0. 1 ± 0.1
18:3n3	6.2 ± 0.6	2.3 ± 0.7	7.5 ± 1.3	7.6 ± 1.3	3.2 ± 0.1	4.1 ± 0.3
20:3n3	11.1 ± 1.1	5.7 ± 0.3	1.9 ± 06	0.8 ± 0.2	0.7 ± 0.1	0.9 ± 0.1
20:3n6	1.1 ± 0.2	1.1 ± 0.1	1.4 ± 0.2	1.1 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
20:4n6	1.6 ± 0.2	2.9 ± 0.2	3.1 ± 0.5	1.5 ± 0.3	5.1 ± 0.4	4.1 ± 0.1
20:5n3	1.2 ± 0.2	2.5 ± 0.8	8.8 ± 1.8	6.9 ± 1.5	12.1 ± 0.2	10.9 ± 0.4
22:6n3	6.4 ± 0.6	2.4 ± 1.1	10.1 ± 2.9	6.8 ± 3.3	15.3 ± 0.5	10.8 ± 1.2
ΣPUFA	37.1 ± 0.9	30.3 ± 2.2	38.8 ± 4.8	31.1 ± 5.5	44.1 ± 1.1	39.1 ± 1.1
EPA/ARA	0.8 ± 0.1	0.9 ± 0.3	2.9 ± 0.5	4.6 ± 0.9	2.4 ± 0.2	2.7 ± 0.1
DHA/EPA	5.2 ± 0.9	0.9 ± 0.1	1.1 ± 0.2	0.9 ± 0.2	1.3 ± 0.1	1.0 ± 0.1

Figure 1. Winter flounder (*Pseudopleuronectes americanus*) juvenile survival (in percent) for the different weaning periods (at settlement [W0]; one [W1], two [W2], and three [W3] months after settlement). Results are expressed as mean ± S.D. Day 30: $F = 16.97$, $P < 0.001$; Day 60: $F = 11.31$, $P < 0.01$; Day 90: $F = 13.39$, $P < 0.01$; different letters indicate significant differences among treatments on the same sampling day.

Figure 2. Non-metric multi-dimensional scaling of the Bray-Curtis similarity matrix based on the profiles of relative abundance of fatty acids associated with commercial inert food, enriched rotifers (*Brachionus plicatilis*), and the neutral and polar lipids fraction of weaned winter flounder (*Pseudopleuronectes americanus*) juveniles two or three months after settlement.


W0
W1
W2
W3
Survival (%)
0
10
20
30
a
b
b
b
a
b
b
a
b
b
30
60
90
Days after settlement


2D Stress: 0.08
Treatment
Inert food
Rotifers
Neutral two months
Neutral three months
Polar two months
Polar three months