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Determination of apparent protein digestibility of live *Artemia* and a microparticulate diet in 8-week-old Atlantic cod *Gadus morhua* larvae

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ABSTRACT

A technique was developed to determine apparent digestibility coefficients (ADCs) of protein in microparticulate and live feeds for marine fish larvae. The technique is analogous to methods used for larger fish and allows for an *in vivo* measurement of protein digestibility by employing a spectrophotometric protein assay for protein determination and rare earth oxides as inert digestibility markers. Either a microbound microparticulate diet developed in our laboratory or *Artemia* nauplii were fed to 8-week-old Atlantic cod as a single 30-min feeding and fecal solids collected 6 h later. Protein ADCs for the two diets were significantly different ($P = 0.016$) with determined ranges of 47 to 58% and 76 to 86%, (*Artemia* and microparticulate diets, respectively). Measured ADCs are presented as a range to account for the possibility of leaching of protein from the microparticulate diet, and the evacuation (loss) of marker from the live prey during the 30-min feeding. It is suggested that this new technique will assist researchers in selecting experimental larval feeds with the most nutritive potential for extended feeding studies. Detection limits for this technique were determined to be 8.6 μg for protein and 0.020 and 0.038 μg for yttrium (Y_2O_3) and dysprosium (Dy_2O_3) markers, respectively, in collected fecal solids.

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1. Introduction

The use of live prey to rear marine fish larvae is well established (Stottrup and McEvoy, 2003). However, the increasing demand for cultured marine fish has spurred the development of microparticulate diets for use in conjunction with live prey or as a direct replacement. The use of microparticulatediets in larval rearing has several advantages over live prey including consistent availability and quality, the absence of live prey culturing systems, increased nutrient delivery, and the opportunity to formulate diets to support the larval growth of a particular species.

The production of microparticulate diets is not trivial. Marine fish larvae may require diets high in free amino acids, small peptides, or other water-soluble nutrients (Koven et al., 2002; Rønnestad et al., 2003). In extruded feeds, these nutrients can partially dissipate into the surrounding water and become unavailable to the fish. While this is not a particular problem with large pellets, the loss of water-soluble nutrients is substantial in larval feeds due to their small size and high surface area (Kvåle et al., 2006; Langdon et al., 2007; Nicklason and Johnson, 2008). As reviewed recently by Langdon (2003), several processing techniques have been explored to minimize the loss of nutrients from microparticulate diets and the physical characteristics of the resulting diets vary greatly. Because many of these microparticulate diets possess semi-permeable biopolymer surfaces, there is a need to evaluate the *in vivo* availability of essential nutrients in these feeds and compare them with that observed in live prey that have successively been used to rear marine fish larvae.

In vitro methods have been successfully used to contrast nutrient availabilities and optimize diet formulation of larval feeds (Alarcón et al., 1999; García-Ortega and Huisman, 2001; Tonheim et al., 2007). However, if the microparticulate diets being evaluated possess different physical characteristics, there may be differences in nutrient availability that are not adequately assessed by *in vitro* techniques. As reviewed recently by Conceição et al. (2007), the intake, digestion, absorption, and utilization of nutrients in larval feeds have been determined *in vivo* via the incorporation of radioactive markers (Rust et al., 1993; Rønnestad xet al., 2001; Morais et al., 2004a,b; Tonheim et al., 2004; Hovde et al., 2005; Kvåle et al., 2006), visible dyes (Werner and Blaxter, 1980; Önal and Langdon, 2004), differences in stable isotope ratios (Schlechtriem et al., 2004, 2005), and auto-fluorescence (Kelly et al., 2000). Many of these techniques, however, are limited to the use of particular novel feeds or feed components and cannot be widely applied to all types of microparticulate and live feeds.

In the current study, apparent digestibility coefficients (ADCs) were determined for protein in a microbound microparticulate diet and a live prey (*Artemia* nauplii) fed to Atlantic cod larvae using a method analogous to the standard method used for larger fish. The

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determination of ADCs by the use of an inert marker has been shown to be a useful tool in the evaluation of *in vivo* nutrient availabilities in fish feeds (Windell, 1978). Feces can be collected by stripping, anal suction or by removal of solids from the water column (Windell et al., 1978). Ufodike et al. (1995) modified the standard method for larger fish by employing micro-Kjeldahl techniques for protein determinations and successfully determined protein ADCs in four fry feeds fed to 100 mg rainbow trout.

The method described by Ufodike et al. (1995) is not applicable for larvae of many marine fish species due to the smaller size of the larvae and the corresponding smaller amount of feces available for analysis. In the cited study, the authors collected at least 5 mg of dried feces for each nitrogen determination by the micro-Kjeldahl procedure. This is approximately two orders of magnitude more fecal material than was obtained per replicate in the present study. As a result, we introduce a spectrophotometric protein assay, the bicinchoninic acid (BCA) protein assay, for use in protein ADC determinations in the current study.

The oxides of rare earth metals such as yttrium and elements of the lanthanide series have been shown to be particularly useful as inert digestion markers and in the determination of ADCs (Austreng et al., 2000; Storebakken et al., 2000; Sørensen et al., 2002). As they are not commonly found in fish or feed components, they have the additional benefit of allowing for the tracking of feed consumed by the fish (Otterå et al., 2003; Cook et al., 2008). These markers can be easily incorporated into formulated diets and Cook et al. (2008) have successfully incorporated these oxides into live prey such as rotifers and *Artemia* nauplii. The markers were successfully used to measure feed intake of *Artemia* nauplii by Atlantic cod larvae (Cook et al., 2008). The rate marked feed passes through the digestive tract of the larvae can also be measured by collecting fecal solids at timed intervals after the meal.

In this experiment, we evaluated the apparent protein digestibility of a novel microparticulate diet and a live prey (*Artemia* nauplii) for Atlantic Cod larvae. The goal of the research was to demonstrate the usefulness of the proposed modifications to the standard method described first by Windell (1978) to determine protein ADCs for larval feeds for marine fish. Protein ADCs were determined for both feeds by employing the micro-BCA protein assay technique to quantify protein and ICP-OES methods to quantify inert rare-earth digestibility markers in fecal and feed samples. Feed intake, the rate of feed passage through the digestive tract, and the amount of ammonia excreted by the larvae during digestion were also measured to fully evaluate the feed's potential to support larval growth.

2. Materials and methods

2.1. Fish culture and collection of feces

Fertilized Atlantic cod (*Gadus* morhua) eggs were purchased from Northern Cod Ventures (St. John's, NL, Canada) and air shipped to our lab in Seattle. The cod were hatched and reared in a marine recirculation system at the Northwest Fisheries Science Center, Seattle, USA (Scott and Rust, 1996). Water was supplied originally from Puget Sound, Washington, USA. Water temperature was 5–7 °C at hatching and was gradually increased to 11 °C by 2 weeks post hatch. Water temperature fluctuated between 11 and 12 °C thereafter. Salinity averaged 28‰. The levels of dissolved oxygen and ammonia in the system water were 9.5 mg L^{-1} and 0.03 mg L^{-1}, respectively, at the time of the experiment. Water temperature was 11.7 °C at the time of the experiment.

Larvae were fed rotifers (*Branchionus plicatilis*) enriched with *Nannochloropsis* microalgae paste (Reed Mariculture, Campbell, CA, USA) from first feeding until week 6. Larvae were then transitioned to *Artemia* nauplii. Larvae were introduced to the microparticulate diet after 4 weeks of feeding and were co-fed microparticulate diet and live prey until the start of the experiment. Feed was withheld on days 55 and 56 post hatch (594–606 °C days) to allow prior feeds to be evacuated from the larvae. After feeding unmarked live prey on day 57 post hatch (617 °C days), fish were stocked into 8 black 55 L tanks. Three replicate tanks were assigned to each of the two dietary treatments and two tanks were assigned as unfed blanks.

Treatment fish were fed once daily on days 58 and 59 post hatch (629–640 °C days), with marked diet. Dry diet was delivered to each tank in one feeding and initially floated on the water surface. The dry diet gradually hydrated on the water surface, slowly passed through the water column where fish ingested the diet, and uneaten diet settled on the bottom of the tank. No fish were observed to ingest either the floating diet or the settled diet on bottom of the tanks. Live prey were also delivered to each tank in one feeding. Live prey were rinsed and dewatered on a 50 µm nylon screen prior to being fed to the tanks.

Fish were allowed to feed for 30 min. After feeding on day 59 post hatch, fish were collected from each tank with disposable plastic pipettes and transferred into 1 L beakers of clean system water. Average fish weight was 87 mg (wet weight). Full fish (a full bolus was observed through the transparent larvae) were selected from the 1 L beakers and transferred into 50 mL beakers containing 40 mL of clean system water to allow fish to evacuate. Similarly, fish from the unfed treatment were transferred to 1 L beakers of clean system water and then transferred into 50 mL beakers. Each 50 mL beaker contained 3 fish and was referred to as an evacuation sample. Six evacuation samples were prepared for each of the 6 treatment tanks and 10 evacuation samples were prepared for each of the unfed tanks. Sequentially transferring the fish twice to beakers containing clean system water was necessary to reduce excess feed and algal particulate matter in the evacuation samples. Only evacuation samples that experienced no mortality were suitable for further laboratory analysis. A higher number of evacuation samples were prepared for the unfed fish tanks due to higher observed mortality among unfed fish samples in preliminary experiments.

Fed fish were allowed to evacuate in the 50 mL beakers for 6 h in the dark at 12 °C in a temperature-controlled incubator. After the evacuation period, fish were removed from the 50 mL beakers and frozen until further analysis. Fecal solids were collected by filtering the evacuation sample through a 50-micron nylon screen and rinsing the solids with deionized water. Fecal solids were transferred to 20 mL glass scintillation vials, and frozen at −80 °C until further analysis. Filtrates were additionally saved for marker analysis. Filtrates from a representative subset of evacuation samples were filtered through a 0.45 µm mixed cellulose ester (MCE) syringe filter, frozen at −80 °C, and saved for subsequent ammonia analyses as described below.

2.2. Preparation of marked feeds

Artemia cysts harvested from San Francisco Bay, USA were obtained from Argent Laboratories (Redmond, WA, USA) and hatched at our laboratory. Nauplii were dosed with marker immediately before feeding to fish larvae as previously described by Cook et al. (2008). Larvae fed *Artemia* received nauplii marked with Y_2O_3 on day 58 (629 °C days) and nauplii marked with Dy_2O_3 on day 59 (640 °C days). Both markers were obtained from Sigma Chemicals (St. Louis, MO, USA). Nauplii were marked with different rare earth oxides on different days to assess the amount of residual feed in the larval gut from the previous day.

A microbound microparticulate diet for marine fish larvae was prepared at our laboratory and marked with Y_2O_3 for use in the experiment. The raw material base was Atlantic cod muscle with the skin and most of the bones removed. The muscle was homogenized with two parts water in a Waring blender (Waring Products, Torrington, CT, USA) and digested with swine trypsin (Fisher Scientific, Pittsburgh, PA, USA) overnight at 4 °C. The digest was then heated to 70 °C to terminate enzyme activity.

The heated digest was cooled and centrifuged to obtain supernatant (LT) and sediment (SD) fractions. The LT fraction contained highly soluble low molecular weight peptides and amino acids and was concentrated to 56% solids for use as a dietary ingredient. The SD

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Table 1
Formulation of microbound microparticulate diet

Ingredient	Amount (g/100g dry weight)
Cod muscle trypsin digest	
–Sediment fraction	76.20
–Supernatant fraction	3.45
Zebra mussel homogenate	1.23
High DHA/EPA ethyl esters[a]	13.56
AquaGrow DHA microalgae[b]	1.23
Algamac-2000 microalgae[c]	1.23
Choline chloride	0.43
l-methionine[d]	0.09
l-phenylalanine[d]	0.09
Stabilized vitamin C[e]	0.15
Vitamin pre-mix[f]	0.49
Mineral pre-mix[g]	0.12
Yttrium oxide[d]	1.70

[a] 30/20 EE Fish Oil from Ocean Nutrition, Mulgrave, NS, Canada.
[b] From Advanced Bionutrition Corp., Columbia, MD, USA.
[c] From Biomarine, Hawthorne, CA, USA.
[d] Sigma Chemicals, St. Louis, MO, USA.
[e] Stay-C from Argent Laboratories, Redmond, WA, USA.
[f] Abernathy salmon vitamin premix (in Hardy, 1989).
[g] USFWS #3 (in Hardy, 1989).

fraction contained approximately 12% solids and was used as the diet base and binder. The complete formulation for the microbound diet is listed in Table 1. Ingredients were homogenized in a Waring blender, oven dried in sheets, ground, and screened to obtain sized microparticle fractions. The size fraction used for the experiment was 212–425 µm (US #70–US #40 mesh sizes).

2.3. Determination of protein content in diets and feces

Protein was assayed in diet and fecal samples via a micro-BCA method (Pierce Biotechnology, Rockford, IL, USA) in accordance with the manufacturer's instructions with the following modifications. The BCA method is a spectrophotometric technique and a standard curve was generated to correlate absorbance vs protein concentration using graduated dilutions of bovine serum albumin (BSA) in sample buffer. Diet and fecal samples were placed in 20 mL glass scintillation vials with Teflon lined caps and digested with 10 mL of 1 N NaOH for 1 h at 60 °C under mild agitation. Samples were then neutralized by adding 4 mL of 2.5 N HCl and buffered to a pH of 9.2 by adding 2 mL of 0.5 N borate buffer. After the sample had cooled to room temperature, deionized water was added to bring the total sample volume to 20 mL.

Two 1.5 mL aliquots from each 20 mL digested sample solution were then assayed for protein via the micro-BCA technique with 1.5 mL of the manufacturer's “working reagent” and results averaged. Digestion in the “working reagent” was extended to 90 min to further develop color and increase the sensitivity of the technique. Sample concentrations were quantified from spectrometer response as µg protein/mL using the BSA standard curve and results expressed as µg protein by multiplying the sample concentration by the sample volume (20 mL). The method's detection limit was determined as 10 times the standard deviation of the protein responses from the unfed fish fecal samples.

In addition to determining protein content via the BCA method, the protein content of diets was also approximated from nitrogen analysis. Nitrogen content of diets was determined via combustion using a LECO FP-2000 Nitrogen Analyzer (LECO Corporation, St. Joseph, MI, USA) following the Dumas procedure as described by AOAC International (2000). Protein content of the diets was approximated by multiplying nitrogen concentration by a factor of 6.25.

2.4. Determination of marker content in diet, feces, and fish bodies

The rare earth metals, yttrium and dysprosium, were quantified in all samples via inductively coupled plasma-optical emission spectroscopy (ICP-OES) with a Perkin Elmer Optima 3000 Radial ICP-OES (Perkin Elmer Instruments, Norwalk, CT, USA). Wavelengths employed were 371.029 and 353.170 nm for yttrium and dysprosium, respectively. Standards were obtained from Ultra Scientific (North Kingstown, RI, USA). Separate calibration standards were prepared for each of the two sample types with appropriate reagents. Detection limits were determined as 10 times the standard deviation of the analyte responses from the unfed fish samples. The preparation of sample types prior to analysis by ICP-OES follows.

2.4.1. Fish bodies and dried diet samples

Larvae bodies were thawed and ashed overnight in a muffle furnace at 550 °C. Diet samples were dried overnight at 105 °C, weighed into 20 mL glass scintillation vials and similarly ashed overnight at 550 °C. Sample ash was digested at 60 °C for 2 h in 10 mL of an aqueous solution containing 10% (v/v) hydrochloric acid and 10% (v/v) nitric acid. Samples were diluted as appropriate and analyzed via ICP-OES.

2.4.2. Fecal samples

Following determination of protein content via BCA, solvated fecal solids were acidified with concentrated HCl and HNO_3 to final concentrations of 10% (v/v) and digested at 60 °C for 2 h. Samples were diluted 5 fold with deionized water prior to analysis via ICP-OES. Further dilutions were prepared as appropriate.

2.4.3. Filtrate from evacuation samples

In addition to determining the amount of marker in fecal solids, the surrounding water from each evacuation sample was collected and analyzed to account for any marker that may have leached into solution. A matrix effect was observed during initial ICP-OES analyses of these samples due to the seawater present. As a result, the surrounding water samples were diluted 10 fold prior to analysis with fresh deionized water and separate marker standards were prepared that included 10% system seawater.

2.5. Estimating feed composition 30 min after being fed

Diet samples analyzed for marker and protein concentrations as described previously represent initial concentrations. However, it was realized during the experiment that protein and marker concentrations of the diets may change during the 30 min larvae were allowed to feed. Concurrent studies at our laboratory have documented marker evacuation from live prey (Cook et al., 2008) and protein leaching from microparticulate diets (Nicklason and Johnson, 2008) over time. Both the live and microparticulate diets used in this study were evaluated by these researchers. Cook et al. (2008) estimated the amount of marker evacuated from dosed *Artemia* nauplii at 30 min to be 21 and 23% for yttrium and dysprosium oxide, respectively. Less than 1% of the yttrium oxide marker was observed to leach from the microbound microparticulate diet during this interval. Nicklason and Johnson (2008)

Table 2
Protein and mineral content of diets (% dry weight)

Diet	% marker		%Crude protein	
	Dy_2O_3	Y_2O_3	BCA	% N×6.25
Initial concentrations				
Artemia (day 58)		9.2	27.3	52.6
Artemia (day 59)	10.1		29.2	48.9
Microparticulate		1.70	52.5	70.4
*Concentrations at end of 30-minute feeding event**				
Artemia (day 58)		7.4	28.0	54.0
Artemia (day 59)	8.0		30.0	50.2
Microparticulate		2.4	43.9	58.8

*Predicted protein and marker concentrations of diets after 30 min in seawater were made from concurrent protein leaching (microparticulate diet) and marker evacuation (live prey) studies at our laboratory (Nicklason and Johnson, 2008; Cook et al., 2008).

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estimated that 40% of the initial protein is lost from this microparticulate diet after 30 min in aqueous solution, but the loss of protein from live *Artemia* nauplii is insignificant after this interval (unpublished data). The expected losses in marker and protein content were used in the determination of diet protein and marker concentrations at 30 min (Table 2). Similar high rates of protein leaching have been observed recently in agglomerated and heat coagulated larval cod diets by other researchers (Kvåle et al., 2006).

2.6. Calculation of apparent digestibility coefficients (ADCs)

After protein and marker contents were determined in diet and fecal samples, protein ADCs were calculated for each fecal sample from the following formula.

$$\mathrm{ADC} = 100\% \times \left[1 - \left(\frac{\mu g\ protein_{(feces)}}{\mu g\ marker_{(feces)}} \times \frac{[marker]_{(feed)}}{[protein]_{(feed)}}\right)\right]$$

Two ADCs were calculated for each sample, one ADC was computed with the initial concentrations of protein and marker in the diet and another ADC was computed with the expected concentrations that would be in uneaten feed after 30 min under conditions of protein leaching (microparticulate diet) or marker loss (*Artemia*). As the actual timing of when larvae consumed feed during the 30-min feeding event was unknown, mean ADC results for each diet are presented as a range of expected values.

2.7. Calculation of feed ingested during a single meal

Feed intake was expressed on a weight percentage basis (dry weight of feed/ wet weight of larvae x 100%). The amount of feed consumed during the 30-min feeding event was determined for each evacuation sample by the following formula.

$$\text{Feed intake} = \frac{(\mu g\ \text{marker}_{(\text{evacuated})} + \mu g\ \text{marker}_{(\text{bodies})})}{[\text{marker}]_{(\text{feed})} \times (\text{wet weight of bodies})} \times 100\%$$

$\text{Marker}_{(\text{evacuated})}$ is the sum of marker measured in the fecal solids and the filtrate from each evacuation sample. As with protein ADC determinations, the dynamic nature of diet marker concentrations over the 30-min feeding event necessitated the presentation of mean feed intakes as a range of expected values spanning the theoretical limits of all diet being consumed either immediately or at the end of the feeding interval.

2.8. Calculation of feed passage

In addition to determining the amount of feed consumed in a single meal, it was also possible to determine the percentage of feed evacuated after 6 h by comparing the amount of marker evacuated by the larvae and that remaining in the larvae bodies by the following formula.

$$\text{Feed Passage} = \frac{(\mu g\ \text{marker}_{(\text{evacuated})})}{(\mu g\ \text{marker}_{(\text{evacuated})} + \mu g\ \text{marker}_{(\text{bodies})})} \times 100\%$$

2.9. Determination of ammonia in water samples

Clean system water and the surrounding water from a subset of evacuation samples were assayed for total ammonia (ionized and unionized) via a modified version of the method described by Slawyk and MacIsaac (1972). Five evacuation samples were assayed from each of the unfed, live prey, and microparticulate diet treatments. Analyses were performed by the Marine Chemistry Lab at the University of Washington. Results are expressed as $\mu g\ L^{-1}\ NH_3$.

2.10. Statistical analyses

For this experiment, tank means were employed as the units of replication for the statistical analyses of ADCs, feed ingested, and feed passage rate. Individual evacuation samples were employed as the units of replication for the statistical analysis of ammonia results. Analysis of variance (ANOVA) determinations were performing using StatView Version 5.0.1 software (SAS Institute, Cary, NC, USA) to elucidate significant differences in ADCs, amount of feed ingested, feed passage rate, and ammonia excreted between dietary treatments. The arcsine transformation described by Zar (1999) was applied to proportionate data prior to ANOVA determinations.

3. Results

3.1. Fish mortality

After 6 h, no mortality was observed among the evacuation samples containing fish fed either the microparticulate diet or live prey and all samples were suitable for subsequent protein and ammonia analyses. In contrast, a high incidence of mortality was observed among evacuation samples containing unfed fish making several samples unusable for protein and ammonia determinations. From one replicate tank of unfed fish, 5 of the 10 evacuation samples experienced no mortality after 6 h and were suitable for protein and ammonia analyses. A higher incidence of mortality was observed among the evacuation samples from the other tank of unfed fish so the evacuation period was shortened to 4 h. After 4 h of evacuation, only 4 of the 10 evacuation samples experienced no mortality and were suitable for protein analyses. The shorter time allowed for evacuation precluded these 4 samples from subsequent comparisons of excreted ammonia.

3.2. Protein and marker analyses

Using the micro-BCA method, protein was quantified in all but 1 fecal sample from fed fish. This exception was due to a very small amount of fecal material collected from one of the evacuation samples containing fish fed live prey. Marker was successfully quantified in all fecal samples using the ICP-OES procedure. From the unfed fish samples, the detection limit for protein analysis by the BCA method was determined to be 8.6 µg. Detection limits for marker analysis were similarly determined as 0.020 and 0.038 µg for the yttrium and dysprosium markers, respectively. Marker and protein concentrations of the live and microparticulate diets are listed on a dry weight basis in Table 2. Also included in Table 2 are the expected marker and protein concentrations of the diets at the end of the 30-min feeding event. The protein content of the live prey determined by the BCA method differed greatly from that determined by nitrogen analysis. Results from the BCA method were used in the calculation of protein ADCs.

3.2.1. Amount of marker leached from fecal solids

The high sensitivity of the ICP-OES procedure allowed for the measurement of marker in both fecal solids and the surrounding water for all evacuation samples. As a result, it was possible to measure the amount of inert digestibility marker leached from the fecal solids for each diet (Fig. 1). The relative percentage of marker retained in fecal solids was similar between the two diets. For the live prey, 94.6±0.6% of the evacuated marker was detected in the solid phase and 5.4±0.6% was detected in the surrounding water from the evacuation sample. For the microparticulate diet, 94.2±1.5% of the evacuated marker was detected in the solid phase and 5.8±1.5% was detected in the surrounding water from the evacuation sample.

3.3. Apparent digestibility of protein in diets

Due to the dynamic nature of the marker and protein concentrations of the diets during the 30-min feeding event, mean protein ADCs

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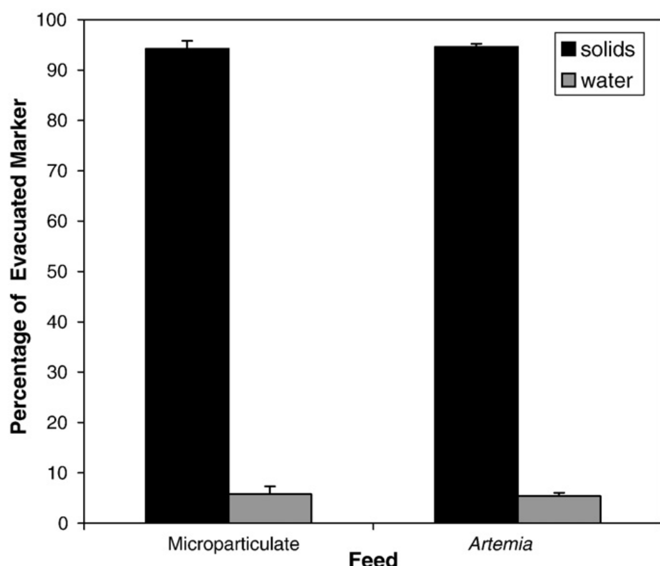

Fig. 1. Amount of inert marker leached from fecal solids. Results are expressed as percentage of total marker excreted in evacuation sample (Average+SEM). "Solids" refers to collected fecal solids and "liquid" refers to the surrounding water from the evacuation sample.

were expressed as a range of expected values for each of the two diets. Range limits reflect ADCs calculated by assuming theoretical instantaneous feedings at the start and at the end of the feeding interval. A protein ADC range was determined for each evacuation sample and tank averages are listed in Table 3. Mean protein ADC ranges for the microparticulate and the live diets were determined as 76–86% and 47–58%, respectively (Fig. 2). Significant differences in the apparent digestibility of protein were observed between the microparticulate and live diets at both the initial (P=0.0012) and final (P=0.0155) theoretical limits.

3.4. Amount of feed ingested during a single meal

As only larvae with a full bolus were selected for evacuation, results represent feed intake by actively feeding larvae. Mean feed intake was 0.57–0.79% and 0.67–0.84% for the microparticulate and live diets, respectively (Fig. 3). There was no significant difference between treatments in feed intake at either the initial (P=0.532) or final (P=0.212) theoretical limits.

Table 3
Apparent digestibility coefficients (ADCs) of protein in diets*

Tank #	ADC (%) Initial	Final
Live Prey (Artemia nauplii)		
1	38	52
2	46	58
3	55	65
Average±SEM	47±5	58±4
Microparticulate diet		
4	85	75
5	89	81
6	84	73
Average±SEM	86±1	76±2

*Initial values determined from diet compositions at the beginning of the 30-min feeding. Final values determined from diet compositions at the end of the 30-min feeding. As feed was consumed by larvae throughout the 30-min feeding, Initial and Final ADCs values define the range of possible apparent digestibilities for diet protein.

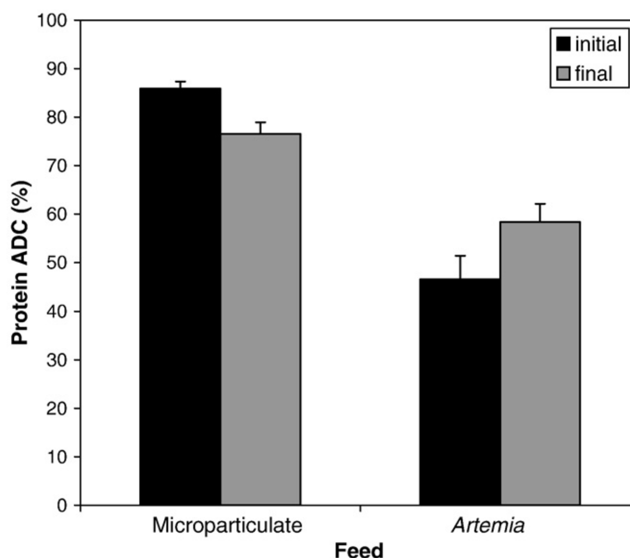

Fig. 2. Apparent digestibility coefficients (ADCs) for diet protein using diet concentrations at the beginning (initial) and end (final) of 30-min feeding (Average+SEM).

3.5. Feed passage

Feed type had an effect on feed passage (P=0.0543) with the microparticulate diet passing through the larvae faster. On average after 6 h, larvae fed the live prey had evacuated 19.1±3.0% of the meal in contrast with those fed the microparticulate diet which had evacuated 32.2±3.7% (Fig. 4). The digestion marker detected in the fecal solids from larvae fed the live prey was almost exclusively dysprosium (99.8±0.1%). The absence of yttrium marker in these samples suggests that virtually all of the *Artemia* nauplii fed on day 58 (629 °C days) had been evacuated in the 24 h prior to feeding on day 59 (640 °C days).

3.6. Ammonia excreted by larvae

The amount of ammonia excreted by the larvae during the 6 h evacuation event was significant and could easily be detected in the surrounding water from all 15 evacuation samples assayed for ammonia. The ammonia concentration in clean system seawater was low and

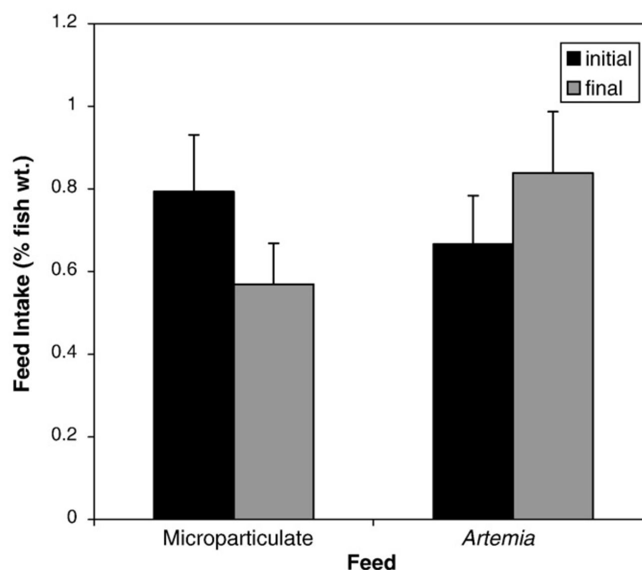

Fig. 3. Feed intake of full fish larvae selected for evacuation expressed as percent dry weight feed/ wet weight larvae (Average+SEM).

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averaged 33.3±0.3 µg L^{-1} NH_3. Levels of ammonia excreted by the larvae were significantly different among all treatments ($P<0.0001$). Mean increases in ammonia concentrations of the surrounding water were 850±30, 1270±30, and 1830±60 µg L^{-1} NH_3 for the unfed, microparticulate diet, and live prey treatments, respectively. Within a feed treatment, increases in ammonia concentration were roughly correlated to feed consumed ($r^2=0.70$ and $r^2=0.57$, Fig. 5).

4. Discussion

The BCA technique is a spectrophotometric method first described by Smith et al. (1985). It is a modified biuret procedure similar to that described by Lowry et al. (1951) and is based on the principle that peptide bonds and some aromatic amino acids will reduce cupric ions (Cu^{+2}) to cuprous ions (Cu^{+1}) in an alkaline environment. Bicinchoninic acid forms an intense purple complex with cuprous ions that can be quantified spectrophotometrically. Wiechelman et al. (1988) observed that, similar to the Lowry technique, dipeptides will not reduce Cu^{+2} ions via oxidation of the peptide bond and that at least a tripeptide is required for this reaction. Because of this, any sample digestion procedure that partially hydrolyzes proteins to dipeptides or free amino acids (FAAs) will reduce spectrometer response. The BCA procedure was selected over that by Lowry due to its increased sensitivity accompanied with reduced interference from the ammonium ion (Smith et al., 1985).

The BCA technique was extensively tested on larval fish feed and fecal samples at our laboratory and found to yield highly reproducible protein determinations. In addition to *Artemia* nauplii and the microbound microparticulate diet employed in the current study, the method was successfully used to determine protein content in rotifers, particle assisted rotational agglomeration (PARA) feeds, and extruded feeds for larger fish (data not shown). While large differences in response were observed between different feed protein, the BCA method demonstrated adequate levels of precision with coefficient of variations of 6% or below.

The reproducible digestion of protein from larval feed and fecal samples is not trivial. There is a trade off between completely solvating sample protein and hydrolyzing peptide bonds. Digestion at lower temperatures, for shorter periods, or with more dilute solutions of alkali resulted in incomplete solvation of proteins in some microparticulate types, but hydrolysis was limited. Conversely, the use of higher temperatures, longer periods of digestion, or higher concentrations of alkali resulted complete solvation of protein but the spectrometer response was decreased due increased hydrolysis of peptide bonds. The proposed digestion parameters represent a compromise between complete solvation and hydrolysis of protein in the microparticulate feeds examined. We observed for several microparticulate types including PARA particles, lipid spray beads, and microbound diets, that digestion of the feeds at 60 °C with 1 N NaOH under mild agitation results in an increase in a spectrometer response over time until approxximately 1 h, where response plateaus and then gradually decreases. Subsequently, a digestion period of 1 h was selected for this study. Response from the BCA technique for samples digested with these parameters was reproducible and could be used to quantify the protein present in fecal samples.

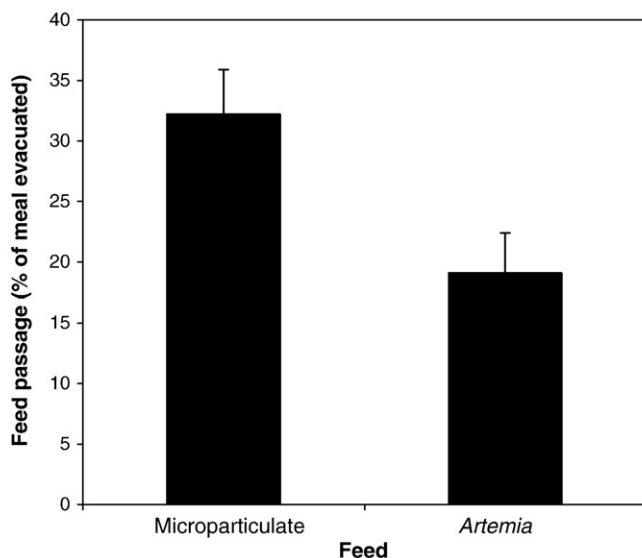

Fig. 4. Feed passage expressed as percentage of meal evacuated by larvae in the first 6 h following the meal (Average+SEM).

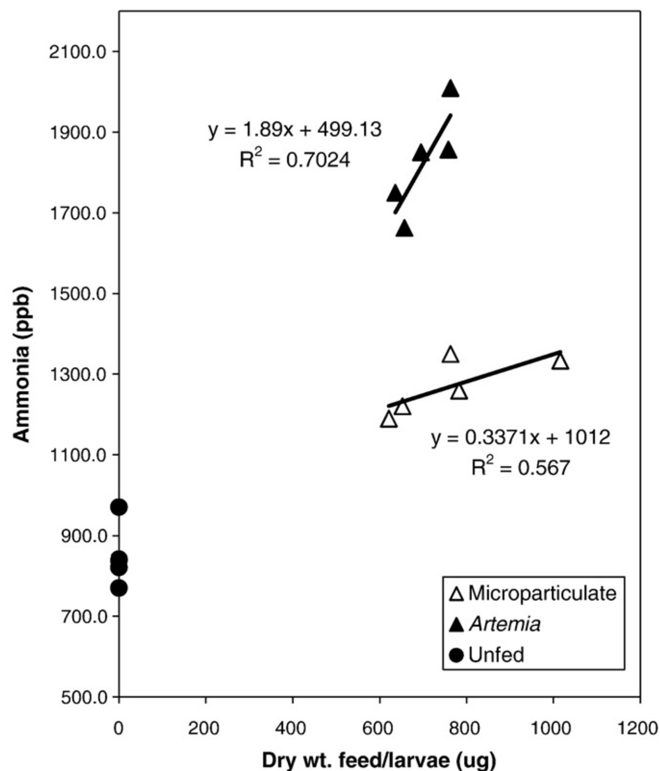

Fig. 5. Ammonia excreted by larvae by feed type. Results are expressed as µg L^{-1} NH_3 in the evacuation sample water vs. average amount of feed consumed per larva on a dry weight basis.

As observed in Table 2, the protein content determined in the live prey and the microparticulate diet varied greatly with the analytical method used. This discrepancy between protein content determined from nitrogen content and that determined from spectrophotometric techniques has previously been observed in live prey (Øie et al., 1997; Srivastava et al., 2006; Kvåle et al., 2007) and is most likely due to the presence of non-protein nitrogen in the live prey organisms and differences in spectrometer response between feed proteins and commonly employed protein standards such as BSA. Protein determinations for *Artemia* nauplii in this study (Table 2) are in agreement with previously reported results from spectrophotometeric (Helland et al., 2003; Aragão et al., 2004) and nitrogen determinations (Watanabe et al., 1983; García-Ortega et al., 1998; Hamre et al., 2002).

Free amino acids may constitute as much as 14% of the nitrogen balance in newly hatched *Artemia* nauplii (Helland et al., 2003). While protein ADCs computed from spectroscopic techniques or from nitrogen determinations should be similar for formulated feeds for larger fish, the choice of the BCA technique to determine protein ADCs of larval feeds is arguably more appropriate due to the higher levels of FAAs and dipeptides present in these feeds. Dietary free amino acids and dipeptides are important for proper larval nutrition (Rønnestad et al., 2003); however, measurements of their uptake may be more meaningful when evaluated separately from dietary protein due to their high

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degree of assimilation. A disadvantage of the BCA technique is the inability to assay protein which has leached from fecal solids into the surrounding water due to spectral interferences from ammonia and urea (Smith et al., 1985). Since larvae feeds are prone to leaching, ADCs may be overestimated due to undetected fecal protein that has leached from the fecal pellet.

In this study, fecal solids were separated from the surrounding water by filtering through a 50-micron nylon screen. This procedure was selected because the fecal pellets were intact and could be easily rinsed from the nylon screen into a vial for digestion with alkali. From visual observation, it appeared that the majority of fecal solids were collected. The majority of the inert digestion marker was detected in the solid fecal fraction rather than the surrounding water, which confirms this observation. This collection procedure would not be appropriate if the evacuated fecal solids were fragile or quickly dispersed in the surrounding water. As the amount of marker leached from fecal material was minimal for both feeds, only marker detected in the fecal solids was used for protein ADC calculations; however, marker detected in the surrounding water was included in feed intake and feed passage calculations.

Based on protein ADC determinations, the digestibility of protein was significantly higher for the microbound microparticulate diet than for the live prey. This demonstrates the potential of this feed for rearing Atlantic cod larvae. As the developed method could be easily modified to return the evacuated larvae back to rearing tanks, protein ADCs of the feeds could potentially be measured over the course of an extended feeding trial. This would provide a better evaluation of the long-term nutritive benefits from the developed feed. Other factors such as feed intake (Teshima et al., 2000), amino acid requirements (Conceição et al., 1997; Rønnestad et al., 2003; Aragão et al., 2004), and adverse effects of prolonged feeding (Baskerville-Bridges and Kling, 2000) should also be evaluated to completely assess the potential of this microparticulate diet to meet protein requirements of fish larvae.

The range of expected protein ADCs for *Artemia* nauplii was determined to be 47 to 58% at 6 h after feeding. This is in agreement with results from Morais et al. (2004a) who found the combined protein and FAA digestibility from radiolabeled *Artemia* nauplii to be 60% in herring larvae and is slightly lower than that observed in a later study with Senegalese sole (Morais et al., 2004b). In the later study, the authors measured combined protein and FAA digestibilities of 77 and 83% at 12 and 35 days post hatch, respectively (Morais et al., 2004b). In the studies by Morais et al. (2004a,b) protein digestibility was determined at 24 h after feeding and larvae had almost completely evacuated the meal. Differences between the studies were attributed to morphological differences between the digestive tract of the two species at the age of the studies, with the digestive tract of the herring larvae being less developed.

It was anticipated that the digestive tract of 59 days post hatch Atlantic cod larvae used in this study would be well developed and protein digestibilities would be more similar to those observed in Senegalese sole than herring larvae. The slightly lower digestibility results determined in the current study can partially be explained by three major differences between the studies. First, digestibilities in the current study were obtained at 6 h after feeding when only 19.1 ± 3.0% of the consumed *Artemia* nauplii had been evacuated. As noted by Morais et al. (2004b), digestibilities determined at 24 h after feeding when no subsequent feed is introduced may overestimate actual nutrient digestibilities in actively feeding larvae due to the longer retention of the feed in the digestive tract. Fecal material excreted in the first 6 h after feeding would be less digested than that excreted later although the former may be a better approximation of the digestibility of *Artemia* nauplii protein in actively feeding larvae.

Secondly, protein and FAA digestibility measurements were combined in the studies by Morais et al. (2004a,b). Free amino acids are known to be highly assimilated by fish larvae (Rønnestad et al., 2003) and protein digestibility measurements would be elevated when FAAs are included as protein. Lastly, the *Artemia* nauplii used in the current study were heavily dosed with inert digestibility marker. The digestive tract of most animals was observed to be completely filled with marker. Any dietary benefit transferred to larvae from gut contents of the live prey was minimal and the determined protein ADC for *Artemia* nauplii in the current study is perhaps more reflective of that from starved nauplii than from enriched animals as were employed in the studies by Morais et al. (2004a,b). Antidotal evidence from several nutritional studies suggest that enriched live prey impart significantly higher nutritional benefits to marine fish larvae than starved live prey and it is reasonable to expect lower protein digestibility to be observed when using starved animals due to the increased percentage of structural protein.

García-Ortega and Huisman (2001) and Tonheim et al. (2007) measured the digestibility of *Artemia* nauplii protein using *in vitro* techniques and reported high protein digestibilities (78 and 84%, respectively). There are several differences between these studies and the current study which make direct comparisons of results inappropriate. In the studies by García-Ortega and Huisman (2001) and Tonheim et al. (2007), nauplii are digested with a cocktail of digestive enzymes found in marine fish larvae and nitrogen balances between soluble and insoluble fractions were compared. As mentioned previously, there is a significant amount of non-protein nitrogen in *Artemia* nauplii which is highly digestible and protein digestibilities which are based on nitrogen balance would be expected to be higher than those based on protein balance. Additionally, protein digestibility values reported by García-Ortega and Huisman (2001) and Tonheim et al. (2007) are from enzymatic digestions at conditions that mostly likely were different from those present in the gut of larvae in the present study. In particular, it would be expected that the pH of the gut of fully fed fish in the present study immediately after feeding would be higher than the expected physiological value due to the large amount of feed consumed. Consequently, enzymatic activity and protein digestibility would be expected to be lower in the present study. Lastly, metabolic protein loss due to the sloughing of intestinal epithelial cells during digestion is included in fecal protein determinations in the current study. As *Artemia* nauplii are especially segmented animals, the loss of intestinal epithelial cells may be significant during digestion. *In vitro* protein digestibilities do not account for metabolic protein losses and results are expected to be higher than those determined by *in vivo* techniques.

On a dry weight basis, the microparticulate diet fed larvae selected for evacuation had consumed slightly more feed than those fed the live prey. This appeared to be due primarily to the lower moisture content of the formulated diet. Larvae fed the live prey appeared to consume a larger volume of feed and several larvae had a distended abdomen after feeding which was not observed among the microparticulate diet fed larvae. This higher consumption demonstrates the potential of the microparticulate diet to deliver more nutrients to the larvae in a single meal due to the lower moisture content. However, it should be noted that we selected larvae that appeared full after feeding for the current study. The values presented represent the amount of feed consumed by full fish and do not represent the average amount of feed consumed by all fish. Many larvae fed the microparticulate diet did not feed at all and a higher percentage of the larvae fed the live prey appeared fuller than those fed the microparticulate diet.

While feed passage varies greatly with the amount of feed consumed and the frequency of feeding, this study showed the feed passage among larvae fed the microparticulate diet was significantly higher than that observed with larvae fed the live prey. This was expected due to the compact nature of the microparticulate diet versus the extended exoskeleton and appendages of the live prey. Because this higher feed passage was accompanied with higher protein content and a higher ADC for protein, it is anticipated that if consumed, the microparticulate diet should better meet the protein requirements of Atlantic cod larvae than the live prey.

The ammonia content of the surrounding water varies with feed type and between fed and unfed fish. Ammonia is the major constituent of nitrogenous excretion in most teleost fish and larvae (Jobling, 1994; Wright and Fyhn, 2001). In a subsequent experiment with antibiotic dosed water, it was determined that the increase in ammonia content

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was due to ammonia being excreted by the larvae and not from microbial digestion of fecal material. As observed by other researchers, fish that have recently eaten a meal excrete significantly more ammonia than unfed fish (Brett and Zala, 1975; Lied and Braaten, 1984). A linear relationship was observed between amount of feed consumed and ammonia excreted for each feed type.

The higher protein ADC and protein content of the microparticulate diet coupled with similar feed intakes and a higher amount of feed evacuated among evacuation samples, demonstrates that on average, the microparticulate diet fed larvae digested more protein then those fed *Artemia* nauplii in this study. The observation that more ammonia was excreted from larvae fed *Artemia* nauplii was unexpected. This suggests that other undetected nitrogen containing end products such as urea may be present in higher amounts in the surrounding water of fish fed the microparticulate diet. While urea is a minor component of nitrogen excretion is most teleost fish (Wright and Fyhn, 2001), Chadwick and Wright (1999) observed that urea can constitute as mush as 100% of the nitrogenous waste from Atlantic cod embryos and 17% of the nitrogenous waste from adult fish. It is also reasonable to suspect that the higher amount of free amino acids present in *Artemia* nauplii may have been rapidly catabolized to meet energy requirements and subsequently resulted in an earlier excretion of ammonia than observed with the microparticulate diet. Lied and Braaten (1984) observed that ammonia excretion peaks around 5–6 h after a meal in young Atlantic cod which is at the time of sampling in the current study. As different feeds can result in a shift in the timing of nitrogen excreted as ammonia (Ribeiro et al., 2005), ammonia levels may have continued to rise in the samples containing the microparticulate diet fed fish and potentially surpassed levels observed from samples containing the *Artemia* fed fish had the experiment continued past 6 h. Lastly, the difference in ammonia excretion between the two feeds may reflect a better balance of amino acids in the microparticulate diet for Atlantic cod larval growth. When amino acids are present in excess of nutritional requirements, fish will catabolize the excess for energy which results in higher levels of nitrogenous excretion than when amino acids are assimilated into protein (Jobling, 1994).

The fish larvae used in this study were of relatively large size (87 mg wet weight), however, the authors feel the method can be applied to smaller larvae. The limiting factors appear to be the ability of the larvae to form an intact fecal pellet and whether the amount of protein present in fecal samples is above the 8.6 μg detection limit for the BCA procedure. In the current study, 3 fish were used per evacuation sample and all but 1 fecal sample were above the detection limit for protein. To apply the method to smaller larvae, more individuals would be needed per evacuation sample and a finer mesh screen would be needed to collect fecal solids. The large number of individuals required for measurements with very small larvae might seem formidable; however, the non-lethal nature of the ADC determination part of the method allows for repeated measurements with the same cohort of fish.

As described previously, the microbound microparticulate diet used in this study was prepared as a wet blend and ingredients were homogenized in a high-speed mixer. Moisture content of the wet blend exceeded 80% and the digestibility marker, Y_2O_3, was well homogenized throughout the diet. The diet was later oven dried in thin sheets prior to grinding and sieving which improved the adhesion of marker to the insoluble protein base. Using the wet blend technique, we have observed 212–425 µm size class feed particles to release less than 1% of the digestibility marker into the tank water during the 30 min feeding interval despite leaching over 40% of the protein. As a result, we were able to assume the amount of marker that had leached from the microparticulate diet into tank water before consumption by the fish larvae was insignificant in the current study. In a subsequent study, we found that a microparticulate diet marked with the same marker, but prepared by dry blending ingredients and not oven drying, resulted in particles with a significant (over 18%) loss of marker into the tank water. In the latter study, the assumption that marker does not leach from the microparticulate diet during the 30 min feeding interval was not valid and subsequent leaching studies were required to determine the composition of the feed pellet after 30 min in seawater.

It has been noted that in studies with larger fish the digestibility marker may be non-uniformly concentrated within the animal and fecal material collected at a single event may not represent a homogenous concentration of marker in fecal solids, yielding erroneous results. We believe that this may be more of a factor for the microparticulate diet than the live prey as a significant number of *Artemia* nauplii were consumed by the larvae during the 30 min feeding event and the exoskeleton of the nauplii partially compartmentalizes the marker through the digestive tract of the larvae. In larger fish, this difficulty can be overcome by increasing the period of fecal collection. In the current study, we attempted to minimize this error by incorporating marker into the feeds in high concentration and performing six separate evacuation collections per tank and averaging calculated ADCs to obtain a single result for each tank. Target concentration of the marker in the microparticulate diet and live prey was 2% by wet weight. Tank mean values where subsequently used as the units of replication in our statistical analysis of results. An alternative approach may be to perform collections on subsequent days with the same cohort of fish and pool fecal material as the methods used to determine protein ADCs are non-lethal.

5. Conclusion

This study presented a relatively simple technique that can be used to compare the *in vivo* digestibility of protein in microparticulate diets and live prey for marine fish larvae. The use of an inert marker not typically present in larval diets additionally allowed for the quantification of the amount of feed consumed and the feed passage. Combined, these techniques should prove useful in the evaluation of new microparticulate diet types and feed formulations for marine fish larvae feeds as wells as assist in the selection of the most promising feeds for long term studies.

The digestibility of protein in the microbound microparticulate diet prepared at our laboratory was higher than that observed for *Artemia* nauplii in eight-week-old Atlantic cod larvae (76–86% vs. 47–58% ADC). The amount of feed ingested by full fish in a single meal was similar for both diets. The feed passage of the microparticulate diet was higher than that observed for the live prey.

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