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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

Ronald B. Johnson [a,*], Matthew A. Cook [b], Peter M. Nicklason [b], Michael B. Rust [a]

[a] Resource Enhancement and Utilization Technologies Division, National Marine Fisheries Service, Northwest Fisheries Science Center, 2725 Montlake Blvd. E Seattle, WA 98112, United States

[b] Aquaculture Research Institute, University of Idaho, c/o Northwest Fisheries Science Center, 2725 Montlake Blvd. E Seattle, WA 98112, United States

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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

* Corresponding author. Tel.: +1 206 860 3458; fax: +1 206 860 3467.
E-mail address: Ronald.B.Johnson@noaa.gov (R.B. Johnson).

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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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