

Use of Acid-Insoluble Ash as a Marker in Absorption Efficiency Studies with the Lemon Shark

BRADLEY M. WETHERBEE¹ AND SAMUEL H. GRUBER

Department of Marine Biology and Fisheries
Rosenstiel School of Marine and Atmospheric Sciences, University of Miami
4600 Rickenbacker Causeway, Miami, Florida 33149, USA

Abstract.—The suitability of acid-insoluble ash as a marker in digestive efficiency studies with the lemon shark (*Negaprion brevirostris*) was investigated. Acid-insoluble ash concentration in the experimental diet was increased by adding celite (diatomaceous earth). Acid-insoluble ash was palatable to test animals, mixed homogeneously within the experimental diet, could be easily and precisely quantified in feces and food, and was not produced by lemon sharks. However, acid-insoluble ash content in feces was significantly lower during the first 24 h of fecal production than during subsequent collection periods, and the marker may not have remained uniformly mixed in digesta. Differential passage rates for this marker and food may influence estimates of absorption efficiency. Passage rate of acid-insoluble ash should be investigated in future studies of digestive efficiency when this marker is used.

The ability of an animal to digest and absorb nutrients from food is among the most important variables measured in studies of fish energetics. Measuring this ability is fundamental to understanding growth of both cultured and wild fishes (Gerking 1955; Talbot 1985). A conventional approach to measuring digestive efficiency is to collect all the fecal material produced after consumption of a known amount of food. However, the difficulty encountered and time required to use this method have led to use of inert markers in food. Quantitative feeding and fecal collection are not essential when a marker is used, and fish can live undisturbed under normal culture conditions (Austreng 1978; Talbot 1985). In addition, use of a marker reduces the cost of digestibility studies, makes them more convenient, and may improve the accuracy of digestive efficiency measurements (Nose 1967; Kotb and Luckey 1972). Markers may prove especially convenient for studies with large animals such as sharks, because large quantities of water, food, and feces are involved, and because these animals retain food in their digestive tracts

for prolonged periods of time (Wetherbee et al. 1987; Wetherbee and Gruber 1990).

To be an effective marker, a substance must be palatable, homogeneously distributed within food, and readily measured, and it must have physical properties that make it discernible throughout the digestive process (Kotb and Luckey 1972; Pappas et al. 1973). A marker must also be inert and non-toxic; it must not be digested, absorbed or produced by the animal; it must not affect digestive processes within the alimentary tract; and it must remain intimately mixed with digesta (Schneider and Flatt 1975; De Silva 1985). The most commonly used marker in fish studies has been chromic oxide (Cr_2O_3) (Jobling 1983). However, use of Cr_2O_3 is restricted to specially formulated diets, and Cr_2O_3 has been found to pass through the digestive tract at a rate different from that of food in a number of fish (Bowen 1978; Kaushik and Teles 1985; Lorico-Querijero and Chiu 1989). Other substances used as markers include silica, cellulose, hydrolysis-resistant (or acid-insoluble) organic matter, and hydrolysis-resistant ash (De Silva 1985; Pandian and Marian 1985). Buddington (1980) and De Silva (1985) concluded that hydrolysis-resistant organic matter was the preferred internal marker for digestibility studies with fish. However, there is still some uncertainty about the use of this marker because it is composed primarily of chitin and cellulose, and the digestive tracts of some fish are thought to possess chitinase or cellulase activity (Bowen 1981; Cowey 1981).

Acid-insoluble ash (AIA) is the mineral ash component occurring in diets that is insoluble in vigorous acid digestion. It is invulnerable to enzymatic hydrolysis and has been used in absorption efficiency studies with pigs (McCarthy et al. 1974), poultry (Vogtman et al. 1975), ruminants (Van Keulen and Young 1977), and more recently with fishes (Bowen 1981; De Silva and Perera 1983; Schmitz et al. 1983). The concentration of AIA in food can be increased above the naturally occurring level by adding celite (diatomaceous earth; Atkinson et al. 1984). Use of AIA as a marker has

¹ Present address: Department of Zoology, University of Hawaii, Edmondson Hall, 2538 The Mall, Honolulu, Hawaii 96822, USA.

resulted in the determination of absorption efficiencies that were similar to estimates obtained by the collection of all feces resulting from a meal (McCarthy et al. 1974; Van Keulen and Young 1977; Thonney 1981; Atkinson et al. 1984). However, Buddington (1980) questioned the reliability of using AIA as a marker because ash was absorbed in the digestive tracts of fish. De Silva and Perera (1983) reported high variability in estimates of absorption efficiency using AIA and recovery of greater than 100% ash in feces.

In this study, acid-insoluble ash was used as a reference substance for determining the effects of ration level on absorption efficiency for the lemon shark (*Negaprion brevirostris*). Our objective was to determine the suitability of AIA for use as an inert marker by examining the palatability of experimental diets containing AIA, the homogeneity of AIA in test diets, and the precision and accuracy of AIA analyses. Passage rate of AIA in relation to food and production of AIA in the digestive tracts of lemon sharks were also investigated.

Methods

Eight lemon sharks (66–74 cm long, 1.6–2.1 kg), collected and maintained in the laboratory as described by Wetherbee and Gruber (1990), were used in the experiments. Experimental diets were prepared by mixing 200 g skinless blue runner (*Caranx crysos*) filet, 50 mL distilled water, and 2 g celite for AIA enrichment in a food processor; 7 g agar was used as a binder. Sharks were held separately in compartments (48 × 66 × 230 cm, 600 L) in a 4,800-L aquarium and fed preweighed amounts of food at levels of 2.0, 1.7, 3.4, 2.6, and 4.3% body weight per day, in that order, as described by Wetherbee and Gruber (1993). To examine the consistency of AIA content in food, subsamples were taken from various locations within each food mixture and the AIA contents were analyzed. To determine whether AIA occurred naturally in blue runner, AIA contents of filets were determined after the filets had been dried and ground into a homogeneous powder.

Feces resulting from experimental diets were removed from water in compartments with a short basting tube and a hand-operated bulb. The fecal samples were centrifuged for 5 min at 3,000 revolutions/min, and water was decanted and discarded. Feces produced by an individual shark were continuously pooled during 24-h collection periods. Feces in centrifuge tubes were dried for 72 h in a drying oven at 60°C. Dried feces were

ground to a fine powder with a Wiley mill (20 U.S. mesh), and energy and AIA content were measured in duplicate for fecal material from each 24-h collection period.

Acid-insoluble ash content was determined by ashing approximately 2 g of food or fecal sample in a muffle furnace for 16 h at 550°C. The resulting ash was boiled in 75 mL of 2 M HCl for 5 min and filtered through ashless filter paper (94 grade); the residue was washed with boiling distilled water. The filter paper and residue were ashed for 16 h at 550°C, and the resulting material was regarded as AIA (Atkinson et al. 1984). Mean values of AIA content in food and feces for each feeding level were compared with analysis of variance (ANOVA; Sokal and Rohlf 1981). Average AIA contents of feces collected during each 24-h collection period within feeding trials were compared to detect changes in AIA concentration in fecal matter over time.

Energy content of samples was determined with a Parr semimicro oxygen bomb calorimeter (Parr Instrument Co. 1981). Absorption efficiency was calculated for each shark at each ration directly by the total fecal collection method:

$$100 \frac{E_i - E_e}{E_i};$$

E_i and E_e are the amounts of energy ingested and egested, respectively.

Absorption efficiency was also calculated by the indirect method based on the concentration of energy and AIA in food and feces:

$$100 \left(1 - \frac{\%AIA_{fd} - [E_{fs}]}{\%AIA_{fs} - [E_{fd}]} \right);$$

[E] is energy concentration, fd denotes food, and fs denotes feces.

To ascertain whether AIA residue occurred in digestive tracts of lemon sharks, the sharks were fed a meal of blue runner filets at the conclusion of the final feeding trial and the AIA contents of resultant feces were determined. Loss of AIA due to processing was estimated by comparing the initial weight of a celite sample with the weight recovered after ashing, filtering, washing, and ashing.

Results

Lemon sharks were immediately attracted to and readily consumed the agar-bound cubes of food containing AIA. There was no difference in the feeding and general behavior of test animals when

TABLE 1.—Comparison of average (\pm SD) percent acid-insoluble ash in fecal material collected during three 24-h periods for lemon sharks fed at five ration levels (BW is body weight), and average percentages for pooled ration levels.

Ration (%BW/d)	% acid-insoluble ash in feces during:			P-value (ANO- VA)
	1st 24 h	2nd 24 h	3rd 24 h	
1.7	6.3 \pm 1.2	6.7 \pm 1.1	7.3 \pm 1.2	0.28
2.0	6.1 \pm 1.3	7.2 \pm 1.3	6.3 \pm 1.4	0.96
2.6	3.8 \pm 1.6	6.4 \pm 2.2	5.1 \pm 1.8	0.09
3.4	7.2 \pm 2.4	9.8 \pm 2.5	8.4 \pm 1.7	0.90
4.3	5.8 \pm 2.0	5.6 \pm 2.3	9.7 \pm 2.2	0.06
Mean for 24 h	5.9 \pm 2.0	7.1 \pm 2.4	7.5 \pm 2.1	0.02

they were fed the experimental diet and when they were fed blue runner filets. The concentration of AIA in subsamples of food taken from various locations within food mixtures used for each ration level did not differ significantly between subsamples ($N = 4$, $P = 0.756$). It was possible to measure the concentration of AIA in relatively large numbers of food and fecal samples with a minimum of effort and equipment.

In a control study to examine the magnitude of AIA loss during analysis, an average of 99.7% (SD, 0.08; $N = 7$) of celite was recovered following the initial ashing of known quantities of celite. An average of 98.4% (± 0.48) of celite was recovered after the remaining filtering, washing, and ashing procedures, indicating only 1.6% loss of AIA due to analytical processing.

Replicate determinations of %AIA in fecal samples did not differ significantly (paired t -test, $P = 0.382$, $N = 56$). Blue runner filets contained only minute quantities of AIA (0.18%) and feces of sharks fed blue runner filets also contained very little AIA (0.30%), indicating that there was no marker residue in digestive tracts and that AIA was not produced by lemon sharks.

Although the concentration of AIA in feces tended to be lowest during the first 24-h collection period, it did not differ significantly ($P > 0.05$) between 24-h collection periods within any of the feeding trials (Table 1). However, when %AIA values for all rations were pooled for each collection period, %AIA in feces collected during the first 24 h was significantly lower than in feces collected during the following 48 h (Table 1). This variation in %AIA resulted in variable calculated absorption efficiencies among collection periods, although the differences were not significant ($P = 0.185$). Thus, estimates of absorption efficiency were not influenced by time of fecal collection.

TABLE 2.—Average (\pm SD) absorption efficiencies of energy for lemon sharks fed at five ration levels (BW is body weight) based on the total collection of all feces produced from a meal and average values based on the indirect method in which acid-insoluble ash is used as a marker.

Ration (%BW/d)	Total collection	Acid-insoluble ash marker
1.7	98.9 \pm 0.5	66.5 \pm 3.3
2.0	98.7 \pm 0.7	61.9 \pm 4.3
2.6	98.8 \pm 0.3	83.1 \pm 3.3
3.4	99.3 \pm 0.3	71.0 \pm 3.0
4.3	99.4 \pm 0.2	74.6 \pm 3.2

Absorption efficiencies determined by the indirect marker method during each collection period were pooled and are shown in Table 2.

Estimates of absorption efficiency based on the total collection of feces approached 100% for lemon sharks at all rations (Table 2). These elevated values suggest that complete collection of all feces resulting from meals was not accomplished. Therefore, accuracy of absorption efficiencies calculated by the indirect marker method could not be evaluated against efficiencies determined by the "total" collection of feces.

Discussion

Food containing AIA in the form of celite is palatable and does not seem to affect the behavior of test animals. This is an especially important consideration for sharks and other large carnivorous fishes, which are difficult to maintain and feed in captivity (Gruber and Keyes 1981). The ease with which celite may be uniformly mixed in food was demonstrated by the similarity of AIA content in replicate samples of the experimental diet in this study.

The consistency of AIA concentration in replicate analysis of fecal samples, and the very small loss of AIA during ashing, treatment with HCl, and filtering demonstrated that %AIA may be precisely and accurately determined in digestive efficiency studies. Little effort is required to quantify AIA in comparison to procedures for other markers such as Cr_2O_3 , crude fiber, and hydrolysis-resistant organic matter (Furukawa and Tsukahara 1966; Buddington 1980; De Silva 1985).

Use of a marker that occurs naturally in the diet of test animals is desirable because such a marker is less likely to affect the digestive physiology of the animal (Talbot 1985). The presence of AIA in blue runner filets confirms that AIA (unlike mark-

ers such as Cr_2O_3) is a naturally occurring component of the diet of the lemon shark. Although AIA was present in blue runner filets, the small quantity (0.18%) was not sufficient for reliable estimates of absorption efficiency. Therefore, AIA was added to experimental diets in the form of celite and %AIA in experimental food included celite and a small amount of naturally occurring AIA.

The finding that only minute quantities of AIA (comparable to %AIA in blue runner filets) were detected in feces collected from lemon sharks fed only blue runner filets suggests that the source of the AIA in these feces was blue runner, and that AIA is not produced by lemon sharks. The small quantity of AIA in postexperimental feces also indicates that no marker residue remained in the digestive tracts of sharks after other fecal matter had been egested.

Because lemon sharks are relatively large animals, they must be kept in large volumes of water, and large amounts of food and feces are involved in studies of their digestive efficiency. The difficulty of collecting all feces produced by lemon sharks in our study was demonstrated by the unrealistically high values of absorption efficiency obtained by the total collection method. A major goal of our study was to observe the effects of ration level on absorption efficiency. Although the effects of ration level on absorption efficiency were revealed by use of the indirect method with AIA as a reference substance, they were not evident among the efficiencies calculated from the total collection method. These findings demonstrate some of the obstacles that must be overcome in collecting all feces produced from a meal and illustrate the usefulness of an indirect marker such as AIA.

However, the observation that AIA did not represent a constant proportion of fecal matter throughout the time that feces were sampled indicates that AIA may not have remained uniformly mixed with digesta. When AIA is used as a marker, indirect calculation of absorption efficiencies may vary depending on the time after a meal that fecal samples are collected. Although absorption efficiencies for lemon sharks did not depend on hour of fecal collection, the concentration of AIA in feces did vary with time, and differential passage of AIA and food may occur in future studies that incorporate this marker. Differential passage of food and other commonly used markers such as Cr_2O_3 (Bowen 1978) is one of the main reasons why researchers have investigated the

suitability of other substances as inert markers. This limitation may also apply to AIA.

The results of this study demonstrate that AIA offers several advantages over other reference substances commonly chosen for use in measurement of digestive efficiency. The benefits of using markers in digestive efficiency studies of large animals such as the lemon shark warrant the continued use of inert markers. However, a possible drawback to the use of AIA is that the marker may not remain uniformly mixed with food throughout the digestive process and variable estimates of absorption efficiency may result. Future digestive efficiency studies in which AIA is used as an inert marker should include investigation of differential passage of AIA and food and evaluation of the effects on absorption efficiency calculations that differential passage could cause.

Acknowledgments

This study was aided by Miguel Martinez, John Signor, Enric Cortes, and Jill Scharold. Warren Servatt collected the sharks, and James D. Parrish reviewed the manuscript. This material is based on work supported by the National Science Foundation under grant NSF-OCE8843425.

References

- Atkinson, J. L., J. W. Hilton, and S. J. Slinger. 1984. Evaluation of acid-insoluble ash as an indicator of feed digestibility in rainbow trout (*Salmo gairdneri*). *Canadian Journal of Fisheries and Aquatic Sciences* 41:1384-1386.
- Austreg, E. 1978. Digestibility determination in fish using chromic oxide marking and analysis of contents from different segments of the gastrointestinal tract. *Aquaculture* 13:265-272.
- Bowen, S. H. 1978. Chromic acid in assimilation studies—a caution. *Transactions of the American Fisheries Society* 107:755-756.
- Bowen, S. H. 1981. Digestion and assimilation of periphytic detrital aggregate by *Tilapia mossambica*. *Transactions of the American Fisheries Society* 110: 239-245.
- Buddington, R. K. 1980. Hydrolysis-resistant organic matter as a reference for measurement of fish digestive efficiency. *Transactions of the American Fisheries Society* 109:653-656.
- Cowey, C. B. 1981. The food and feeding of captive fish. Pages 223-246 in A. D. Hawkins, editor. *Aquarium systems*. Academic Press, New York.
- De Silva, S. S. 1985. Evaluation of the use of internal and external markers in digestibility studies. Pages 96-102 in C. Y. Cho, C. B. Cowey, and T. Watanabe, editors. *Finfish nutrition in Asia*. International Development Research Centre, Ottawa.
- De Silva, S. S., and M. K. Perera. 1983. Digestibility

- of an aquatic macrophyte by the cichlid *Etroplus suratensis* (Bloch) with observations on the relative merits of three indigenous components as markers and daily changes in protein digestibility. *Journal of Fish Biology* 23:675-684.
- Furukawa, A., and H. Tsukahara. 1966. On the acid digestion method for the determination of chromic oxide as an index substance in the study of digestibility of fish feeds. *Bulletin of the Japanese Society of Scientific Fisheries* 32:502-508.
- Gerking, S. D. 1955. Influence of rate of feeding on body composition and protein metabolism of bluegill sunfish. *Physiological Zoology* 28:267-282.
- Gruber, S. H., and R. S. Keyes. 1981. Keeping sharks for research. Pages 373-402 in A. D. Hawkins, editor. *Aquarium systems*. Academic Press, New York.
- Jobling, M. 1983. A short review and critique of methodology used in fish growth and nutrition studies. *Journal of Fish Biology* 23:685-703.
- Kaushik, S. J., and A. Oliva Teles. 1985. Effect of digestible energy on nitrogen and energy balance in rainbow trout. *Aquaculture* 50:89-101.
- Kotb, A. R., and T. D. Luckey. 1972. Markers in nutrition. *Nutrition Abstracts and Reviews* 42:813-845.
- Lorico-Querijero, B. V., and Y. N. Chiu. 1989. Protein digestibility studies in *Oreochromis niloticus* using chromic oxide indicator. *Asian Fisheries Science* 2:177-191. (Asian Fisheries Society, Makati, Manila.)
- McCarthy, J. F., F. X. Aherne, and D. B. Okai. 1974. Use of HCl insoluble ash as an index material for determining apparent digestibility with pigs. *Canadian Journal of Animal Science* 54:107-109.
- Nose, T. 1967. Recent advances in the study of fish digestion. Pages 83-94 in J. L. Gaudet, editor. *Symposium on feeding in trout and salmon culture*. European Inland Fisheries Advisory Commission, Rome.
- Pandian, T. J., and M. P. Marian. 1985. Nitrogen content of food as an index of absorption efficiency in fishes. *Marine Biology* 85:301-311.
- Pappas, C. J., O. W. Tiemeier, and C. W. Deyoe. 1973. Chromic sesquioxide as an indicator in digestion studies on channel catfish. *Progressive Fish Culturist* 35:97-98.
- Parr Instrument Co. 1981. *Manual 160*. Parr Instrument, Moline, Illinois.
- Schmitz, O., E. Greuel, and E. Pfeffer. 1983. A method for determining digestibility of nutrients in eels. *Aquaculture* 32:71-78.
- Schneider, B. H., and W. P. Flatt. 1975. The evaluation of feeds through digestibility experiments. University of Georgia Press, Athens.
- Sokal, R. R., and F. J. Rohlf. 1981. *Biometry*, 2nd edition. Freeman, San Francisco.
- Talbot, C. 1985. Laboratory methods in fish feeding and nutritional studies. Pages 125-154 in P. Tytler and P. Calow, editors. *Fish energetics: new perspectives*. Johns Hopkins University Press, Baltimore, Maryland.
- Thonney, M. L. 1981. Acid insoluble ash as a digestion marker. Pages 118-122 in *Proceedings of the 1981 Cornell nutrition conference for feed manufacturers*. Cornell University Press, Ithaca, New York.
- Van Keulen, J., and B. A. Young. 1977. Evaluation of acid-insoluble ash as a natural marker in ruminant digestibility studies. *Journal of Animal Science* 44:282-287.
- Vogtman, H., H. P. Pfrtner, and A. L. Prabucki. 1975. A new method of determining metabolisability of fatty acids in broiler diets. *British Poultry Science* 16:531-534.
- Wetherbee, B. M., and S. H. Gruber. 1990. The effects of ration level on food retention time in juvenile lemon sharks, *Negaprion brevirostris*. *Environmental Biology of Fishes* 29:59-65.
- Wetherbee, B. M., and S. H. Gruber. 1993. Absorption efficiency of the lemon shark *Negaprion brevirostris* at varying rates of energy intake. *Copeia* 1993:416-425.
- Wetherbee, B. M., S. H. Gruber, and A. L. Ramsey. 1987. X-radiographic observation of food passage through the digestive tracts of lemon sharks. *Transactions of the American Fisheries Society* 116:763-767.