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Short communication

Making sense of nonsense: Using regression analysis to deal with highly variable data collected from a yellowtail kingfish (*Seriola lalandi*) digestibility experiment

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ABSTRACT

When conducting digestibility experiments with fish many researchers encounter problems that result in erroneous digestibility coefficients. Erroneous digestibility coefficients result from variability in the raw data which is amplified by the formulae used to calculate them. Variation can stem from natural differences in the digestibility of the same diet between individuals or groups of fish. It can also creep insidiously into studies due to poor preparation and mixing of ingredients or problems with the collection of faecal material or the veracity of analytical results. Therefore, it is fairly common to expect variability in data collected from digestibility trials with fish. But what can be done about it? In this paper, we present an experiment with yellowtail kingfish (*Seriola lalandi*) that was done to determine whether the digestibility of extruded wheat (EW) was affected by its inclusion rate (10, 20, 30 or 40% diet^{-1}). The experiment, based on the indicator–ratio method, was conducted without incident, but the raw data on the nutrient and marker concentration of faecal samples was variable. We dealt with this problem by using linear regression to estimate more reliable analytical values for faecal samples. These values were used to recalculate logical digestibility coefficients for EW. Using this approach, we show that dry matter (≈ 57%–40%), carbohydrate (≈ 48%–27%) and gross energy (57–42%) digestibility of EW decline as its inclusion rate rises, whereas the digestibility of protein (≈ 84%) and the digestibility of fat (≈ 94%), remain reasonably constant. We validated the regression approach applied to yellowtail kingfish by examining published data from a similar digestibility experiment on Australian snapper *Pagrus auratus*. A regression approach was useful in reducing the variability in our raw data because the design of our experiment approximated a dose-response relationship. Designing digestibility experiments using a dose-response approach may prove useful in overcoming the inherent variability often encountered in these types of experiments.

1. Introduction

Data on the apparent digestibility of ingredients by fish is necessary if aquafeeds are to be formulated on a digestible nutrient and energy basis (Booth et al., 2013a; Bureau et al., 1999; Glencross et al., 2007). However, in-vivo experiments designed to determine the digestibility of ingredients by fish are difficult to execute, time consuming and expensive. Most common among the contemporary methods used to measure and calculate apparent digestibility of diets and ingredients is the indicator–ratio method, whereby a non-digestible marker (e.g. chromic oxide or yttrium oxide) is added to a nutritionally complete reference diet and this diet is mixed with the test ingredient of interest. The resulting test and reference diets are then fed to the fish for an appropriate time before faecal material is collected and analysed. Differences in the concentration of the marker, nutrients and energy in the samples of feed, ingredients and faeces are then used to calculate digestibility coefficients for the diets and ingredient, respectively, using well accepted formulae (Bureau and Hua, 2006; Cho et al., 1982; Forster, 1999; Glencross et al., 2007; NRC, 2011). However, the indirect technique of determining digestibility coefficients in fish is highly sensitive to breakdowns in the general assumptions underlying the methodology (Bureau and Hua, 2006; Glencross et al., 2007; NRC, 2011). These assumptions are easily derailed when variability (error) enters experiments via biological (the animal), physical (the researcher) or analytical (chemical analyses) pathways. Unfortunately, these unavoidable errors are carried forward into the formulae used to calculate both diet and ingredient digestibility coefficients. The very nature of the formulae tends to compound and amplify even small errors which

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can result in calculation of erroneous or illogical digestibility coefficients (i.e. coefficients less than zero or > 100%) (Bureau and Hua, 2006; Glencross et al., 2007).

A certain amount of biological variability in digestibility experiments with fish is expected due to inherent differences in the digestive function of individual fish, even when fed the same diet. Superimposed on the purely biological variation is that caused by the water temperature of the experiment, the method of collection, differences in the time that faeces is collected from fish, the size of fish and probably the pattern or frequency of feeding. Of the above, the greatest source of variability comes during the collection of faecal material. Whether collected by settlement, stripping or dissection, each method is prone to particular problems that decrease the chance of collecting a truly representative sample of faeces. Settlement techniques result in leaching losses, stripping techniques are prone to collection of undigested material and urinary products, and dissection results in collection of endogenous material not associated with the faeces as well as the death of the fish (Hardy, 1997).

Physical issues that introduce error into digestibility studies are largely under the control of the researcher and involve the inaccurate weighing, preparation and mixing of ingredients (especially homogeneity of the marker in the diet) and the poor treatment and preservation of samples prior to chemical analysis. A certain amount of variation in analytical results on feeds, ingredients and faeces is expected because of the variability in instrumental techniques and, possibly the skill of the chemist. To some extent, this can be controlled by judicious use of standards or testing duplicate samples. If analyses are done well, the variability expected in analytical results on similar samples should be low. It is clear from the above that there are several ways in which biological, physical and analytical problems can affect the results of a digestibility experiment. Too much variation in the raw data will result in calculation of erroneous ingredient digestibility coefficients that are useless when formulating aquafeeds. But what can be done about it? In some cases it may be necessary to abandon the data and start again, providing resources are available. In other cases the data may be variable, but still worthy of evaluation. There is little in the literature to guide researchers on how to proceed in the latter case.

This paper presents an experiment with yellowtail kingfish (*Seriola lalandi*) that was done to determine whether the digestibility of extruded wheat (EW) was affected by inclusion rate (10, 20, 30 or 40% diet^{-1}). The experiment, based on the indicator–ratio method, was conducted without incident, but the raw data on the nutrient and marker concentration of faecal samples was highly variable resulting in erroneous estimates of digestibility. This problem was dealt with by using linear regression to estimate more reliable values for the faecal samples based on the fact the data conformed to a dose-response relationship. These values were then used to recalculate digestibility coefficients for EW. Using this approach we show that dry matter, carbohydrate and gross energy digestibility of EW decline in response to increasing inclusion, whereas protein and fat digestibility remain reasonably constant. The regression approach was useful in reducing the variability associated with the chemical composition of faecal samples and in understanding how that variability affected calculation of apparent digestibility coefficients. The approach taken with yellowtail kingfish was validated by applying the same technique to a similar experiment with Australian snapper (*Pagrus auratus*) (Booth et al., 2005). The regression approach used in this study may prove useful to other researchers who encounter similar problems with highly variable data.

2. Materials & methods

2.1. Ethics statement

This study was performed under the NSW DPI Fisheries Animal Care & Ethics Research Authority known as 'Aquaculture Nutrition ACEC 93/5–Port Stephens'. Care, husbandry, and termination of fish was carried out according to methods outlined in 'A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research' (ACEC, 2015). A brief summary of the experimental design is presented in Table 1.

Table 1
General overview of the design of the digestibility experiment.

	Parameter
Reference diet	Fish meal based
Extruded wheat level (%)	10, 20, 30 & 40
Experiment tanks	10
Diet replication	2
Fish per tank	3
Mean stock weight (kg)	1.4
Pellet diameter (mm)	8
Diet acclimation period (days)	7
Temperature (°C)	22 ± 1
Dissolved oxygen (mg L^{-1})	7 ± 1
Salinity (‰)	32 ± 1
pH	8 ± 0.5
NH_4^+ (mg L^{-1})	≤ 0.3

2.2. Ingredients and preparation of experimental diets

All ingredients (Table 2) were ground in a hammer mill to a flour-like consistency prior to their inclusion in experimental diets (Raymond Laboratory Mill, Transfield Technologies, Rydalmere, NSW, Australia; 1.6 mm screen). Constituent ingredients, including the inert marker (Cr2O3; MERCK Technipur™, Darmstadt, Germany) were then combined on a dry matter basis and mixed (Hobart Mixer; Troy Pty Ltd., Ohio, USA) according to the five formulas presented in Table 3. Water was then added and the wet mash was formed into 8 mm pellets using a meat grinder (Barnco Australia Pty Ltd., Leichhardt, NSW, Australia). Moist pellets were then dried at ≈ 35 °C until the dry matter content was about 900 g kg^{-1} diet. Finished diets were stored in a freezer.

2.3. Fish stock, handling protocols and faecal collection procedures

Yellowtail kingfish used in the experiment were progeny of wild brood-stock housed at NSW DPI Port Stephens Fisheries Institute (PSFI). Sub-adult fish were lightly anaesthetised (10–25 mg L^{-1} Aqui-S®; Lower Hutt, New Zealand; http://www.aqui-s.com) before being weighed (1.4 kg body weight) and transferred into experiment tanks (200 L cylindrical white polyethylene). Two replicate tanks were assigned to each of the 5 dietary treatments. The laboratory we used contained a total of 20 experiment tanks; however groups of fish were only stocked into every alternate tank. This was done to provide a well oxygenated tank in which to recover heavily anaesthetised fish after stripping procedures. This handling protocol was repeated over consecutive stripping events until enough faecal material was obtained for chemical analysis. Fish were fed twice daily (1030 h and 1530 h) during the acclimation phase and three times daily on the day prior to stripping (i.e. 1030 h, 1530 h; overnight between 1900 and 2000 h using clockwork belt-feeders).

Faecal collection was done in the morning between 0900 h–1130 h after fish had been heavily anaesthetised within their respective experiment tanks (50–60 mg L^{-1} Aqui-S®). Prior to expelling faeces gentle pressure was applied to the abdominal region by running the thumb and forefinger from the pelvic fin to the vent. This was done to expel urinary products and prevent them from contaminating the faecal sample. The area around the vent was then wiped clean and faecal material was expressed into 70 mL sample jars using the same technique. Daily faecal collections from individual tanks were pooled and frozen (< −15 °C). Afterwards, faecal samples were dried at room temperature for 24 h in vacuum desiccators filled with silica desiccant. Dry faecal samples were finely ground (Waring, model 32 BL 80, New