Comparison of faecal collection methods, and diet acclimation times for the measurement of digestibility coefficients in barramundi (*Lates calcarifer*)

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Abstract

This study aimed to investigate the effects of two faecal collection methods (stripping and settlement) on the apparent digestibility coefficients (ADC) of dry matter, protein and energy of three different diets fed to barramundi. In a second experiment, the effect of acclimation time (i.e. number of days fed the diet) on the calculation of ADCs was also investigated. Each tank of fish was fed one of three diets for 12 days. Faeces were collected by both stripping and settlement, though only settlement was used prior to day seven of the acclimation period. Faeces were collected using the settlement method at regular intervals from day one to day 12. Comparisons between faecal collection methods were only made based on faecal material collected over a similar acclimation period. The collection of faeces by stripping produced more conservative ADCs, which were also more consistent than those obtained using the settlement technique. The calculated ADCs typically fluctuated for the first three days of collection before the variability diminished. Barramundi should be acclimated to diets for a minimum of four days before collection of faecal material, and collection by stripping is recommended to obtain the most reliable digestibility data.

Introduction

The basis for sound diet formulation depends on having accurate and reliable data on the digestible nutrient and energy value of raw materials that are used to make those diets (reviewed by Glencross et al., 2007). The determination of the digestible nutrient and energy value of raw materials depends on having a viable method to measure the digestibility of these parameters from the diets (Choubert et al., 1982; Suigura et al., 1998; Weatherup & McCracken, 1998). However, the assessment of the digestibility of aquaculture diets can be highly variable and the digestibility values are known to vary significantly depending on the different methods used (reviewed by Glencross et al., 2007). It is well recognised that faecal collection is an integral part of the process for calculating digestibility values, and the collection process can have a significant effect on the determination of the digestibility values of diets (Windell et al., 1978; Weatherup & McCracken, 1998; Vandenberg & de la Noue, 2001; Glencross et al., 2005).

Faecal collection methods can be grouped under two main methods; collection of un-defecated digesta, and collection of faeces settled from the water column. The three most common techniques to collect un-defecated digesta are intestinal dissection, suction, and stripping (Austreng et al., 1978; Vandenberg & de la Noue, 2001; Glencross et al., 2005; Aslaksen et al., 2007). Collection of faeces from the water column involves either syphoning faeces from the bottom of the tank, collection of decanted faeces, or continuous collection (Choubert et al., 1982; Cho & Kaushik, 1990; Vandenberg & de la Noue, 2001; Glencross et al., 2005).

Collection of un-defecated digesta is generally more labour intensive than collecting faeces from the water column and is also restricted by fish size (i.e. fish can be too small or large to handle). Moreover, samples are collected at one point in time providing a snapshot of the ADC and the amount of sample collected can be limiting. In contrast, the collection of faeces from the water column is typically less labour intensive, and can be applied to fish of any size, and does not inflict stress on the animals (reviewed by Glencross et al., 2007). However, owing to passive nature of this collection method, there is a risk of the sample being contaminated by scales, mucous and other exogenous material as well as leaching of nutrients into the water column (reviewed by Glencross et al., 2007). While each method has advantages and disadvantages, it has been suggested that the collection of un-defecated digesta results in a reduced Apparent Digestibility Coefficient (ADC) values (Vandenberg & de la Noue, 2001; Glencross et al., 2005). Although there have been comparisons of methods for other species, there have been no direct comparisons for barramundi when faeces have

been collected by stripping or settlement methods (Vandenberg & de la Noue, 2001; Glencross et al., 2005; Glencross, 2006).

Most studies allow fish to adapt to new diets before commencement of faecal sampling; with times varying between five days and 14 days for a range of temperate and tropical species (Glencross et al., 2005; Barrows et al., 2007; Glencross et al., 2012). This is done supposedly to allow the fish to adapt to the chemical composition of a new diet and establish an equilibrium within the animals gut in terms of the absorption efficiencies from that new diet before any sampling is initiated. However, although it is widely accepted that fish require a period of time to acclimate to new diets, there have been limited studies published that actually investigate the time that it actually take to adapt to introduction of a new a diet or indeed variable levels of feed intake (reviewed by Glencross et al., 2007). Given the importance of accurately determining the digestibility of diets and raw ingredients, this is an area which requires further attention.

Therefore, the present study was conducted to examine two key methodological issues for digestibility assessment with barramundi (*Lates calcarifer*). In the first experiment, differences in the digestibilities of dry matter, protein and energy of three diets (basal, starch and lupin-meal based) were evaluated after faeces were collected by stripping or settlement methods. In the second experiment, the variability of ADCs were evaluated over the first 14 days when barramundi were introduced to a new diet, using faeces collected by settlement collection methods.

Methods

Ingredient preparation and diet formulation

The experiment design was based on a diet formulation strategy that allowed for the diet-substitution digestibility method to be used (Aksnes et al., 1996). For this, a basal diet was formulated and prepared as one large batch (60 kg) to include approximately 540 g/kg DM protein, 120 g/kg DM fat and an inert marker (yttrium oxide at 1 g/kg) (Table 1). This basal mash was prepared and thoroughly mixed, forming the basis of the experimental diets in this study. Each of the test diets were made by the inclusion of 30% of the test ingredient to a sub-sample of the basal mash.

Two test ingredients were used in this study, pre-gelatinised wheat starch, and *Lupinus angustifolius* cv. Myallie (MKM) (Table 2). The fishmeal was ground using a Mikro-Pulveriser hammer mill through a 500 μm screen (Hosokawa Micron Powder Systems, Summit, New Jersey, USA). The lupin meal was ground using a Retsch^{TM} ZM200 rotor mill (Retsch Pty Ltd, North Ryde, NSW, Australia) such that it passed through a 750 μm screen. The other ingredients were supplied in fine flour (< 500 μm) forms and required no further milling. The composition and source of all of the ingredients used are presented in Table 2.

Each of the diets were processed by addition of water (about 30% of mash dry weight) to the mash whilst mixing to form a dough, which was subsequently screw pressed using a Dolly Pasta Extruder (La-Monferrina, Sant'Ambrogio di Torino, Italy) through a 5 mm diameter die. The moist pellets were then oven dried at 60°C for approximately 24 h and then allowed to cool to ambient temperature in the oven. The basal diet was prepared in a similar manner, but without the addition of any test ingredient.

Fish Handling and Faecal Collection

Juvenile barramundi were kept in an experimental tank array (6 x 300 L) supplied with flow-through seawater (salinity =35 PSU) at a rate of about 4 L min^{-1} and maintained with a dissolved oxygen content of 6.4 ± 0.2 mg L^{-1} at 28.8 ± 0.2°C. Each of the tanks were stocked with 10 fish of an initial weight of 398 ± 69 g (mean ± S.D.; n = 40 from a representative sample of the population). Treatments were randomly assigned amongst the 6 tanks, with each treatment having four replicates, but the experiment being conducted over two block events to achieve this level of replication. The same batch of fish was used for both

blocks, but a complete randomised design applied to each block to ensure experimental validity. The fish were allowed to acclimate to their allocated dietary treatment for at least seven days before stripping faecal collection commenced.

All fish were manually fed the basal diet for 1 week prior to the commencement of the trial. On commencement, the fish were fed their respective diets to apparent satiety as determined by the loss of feeding activity after being offered food on three independent feeding episodes over a ninety-minute period once daily (1530 to 1700), seven days a week. Faeces were then collected the following morning (0830 – 1030) from each fish within each tank using stripping techniques based on those reported by Glencross (2011). Fish were anesthetised using AQUI-S™ (0.02 mL L^{-1}). Once loss of equilibrium by the fish was observed, close attention was then paid to the relaxation of the ventral abdominal muscles of the fish to enable the fish to be removed from the water prior to the faecal pellet being expelled. The faeces were then removed from the distal intestine using gentle abdominal pressure during this muscle relaxation. Hands were rinsed between handling each fish to ensure that the faeces were not contaminated by urine or mucous. Fish were also not stripped on consecutive days in order to minimise stress on the animal (as determined by loss of appetite and physical damage, of which none was observed) and maximise feed intake prior to faecal collection. Faecal samples from different days were pooled within tank, and kept frozen at –20°C before being freeze-dried in preparation for analysis. Faeces were collected from three separate stripping events within one week.

Settled faeces were collected overnight from the same tanks and fish using settlement methods based on those reported by Cho & Kaushik (1990) on days 1, 2, 3, 4, 6, 8, 10, and 12. The collection chamber was flushed 1 hour after feeding to remove any feed partials before a chiller jacket (tube with a frozen block of water inside and a hole to allow for the faecal collection tube to be inserted) was placed over the collection tube. Faeces were removed from the ice-chilled collection tube at 0830 on each day, prior to the fish being stripped, and transferred into a large vial before being stored at -18˚C.

For comparison of faecal collection methods, the stripped faecal data was compared against the data from the last four days of settlement collection so as to ensure that the samples were from a similar period of acclimation to the diets.

Chemical and digestibility analysis

Faecal, ingredient and diet samples were analysed for dry matter, yttrium, nitrogen and gross energy content. All methods were done in accordance with AOAC methodology (2005). In addition, diet and ingredient samples were analysed for ash and total lipids and carbohydrate content calculated. Dry matter content was calculated following oven drying at 105ºC for 24 h. Total yttrium concentrations were determined using inductively coupled plasma mass spectrophotometry (ICP-MS) after mixed acid digestion based on the method described by (McQuaker et al., 1979). Protein was determined based on measurement of total nitrogen by CHNOS auto-analyser, and then multiplied by 6.25. Total lipid content of the diets was determined gravimetrically following extraction of the lipids using chloroform:methanol (2:1). Gross ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h. Gross energy was determined by adiabatic bomb calorimetry. Total carbohydrates were calculated based on the dry matter content of a sample minus the protein, lipid and ash. Amino acid composition of samples was determined by an acid hydrolysis prior to separation via HPLC. The acid hydrolysis destroyed tryptophan making it unable to be determined using this method.

The apparent digestibility (AD_{diet}) for each of the nutritional parameters examined in each diet was calculated based on the following formula (Maynard & Loosli, 1979):

$$AD_{diet} = \left(1 - \left(\frac{Y_{diet} \times Parameter_{faeces}}{Y_{faeces} \times Parameter_{diet}} \right) \right) \times 100$$

where Y_{diet} and Y_{faeces} represent the yttrium content of the diet and faeces respectively, and $Parameter_{diet}$ and $Parameter_{faeces}$ represent the nutritional parameter of concern (dry matter, protein or energy) content of the diet and faeces respectively. Ingredient digestibility values were not determined for the present study.

Statistical analysis

All figures are mean ± SE unless otherwise specified. Effects of diet and collection method were examined by two-way ANOVA. Levels of significance were determined using a Tukey's HSD test, with critical limits being set at $P < 0.05$. Effects of sampling time on the digestibility parameters were also analysed by two-way ANOVA. All statistical analyses were done using the software package Statistica™ (Statsoft®, Tulsa, OA, USA) although graphically presented using Microsoft Excel (Microsoft Corporation, USA).

Results

Faecal collection methods

Faecal collection method (settlement or stripping) affected the digestibility of dry matter, protein and energy ($P<0.05$; Table 3). When faeces were collected by settlement compared with stripping the dry matter digestibilities were higher, but both protein and energy digestibilities were lower.

For faeces collected by stripping, the DM digestibility varied between diets ($P<0.05$) with the digestible DM of the MKM diet being significantly lower than that of the Starch based diet ($P<0.05$; Table 4). Protein digestibility was not different between diets when faeces were collected by stripping ($P>0.05$; Table 4) although energy digestibility differed significantly among each of the diets. The energy digestibility was lowest for the MKM diet compared with the basal and starch diets, and the basal diet energy digestibility was significantly higher than the digestible energy of the starch diet ($P<0.05$; Table 4).

Collection of faeces by settlement displayed similar results, with the digestible DM of the MKM diet being significantly lower than both the basal and starch based diets ($P<0.05$; Table 4). No differences were observed between protein digestibility ($P>0.05$; Table 4), whilst energy digestibility was significantly lower for the MKM diet compared with the basal and starch diets, and the digestibility of the basal diet was significantly higher than that of the starch based diet ($P<0.05$; Table 4).

There was good correlation between both the stripping and settlement faecal collection methods and this can be seen by the high R^2 values in Figure 2. Correlation was strongest with energy digestibility ($R^2=0.979$), followed by dry matter digestibility ($R^2=0.823$) and protein digestibility ($R^2=0.655$).

Temporal variation in digestibility values

Statistically there was no temporal variation ($P=0.148$) or interaction effect ($P=0.517$) with time and diet in the DM digestibility, but it did vary between diets ($P=0.001$; Table 5). Protein digestibility was also different between diets ($P=0.003$), but not over time ($P=0.102$) and again there was no interaction effect ($P=0.700$; Table 5). Energy digestibility differed significantly with diet ($P<0.001$), but not with time ($P=0.346$).In contrast to the other two digestibility parameters the energy digestibility did exhibit an interaction effect between diet and time ($P<0.001$; Table 5).

From Figure 3 it can be noted that the DM digestibility values stabilised between days three and four for all diets. Variance within the DM digestibility values was highest on day 1 and thereafter subsided and for all samples, except the MKM, was minimal from day two onwards. There was a limited amount of variation during the first four days in the protein digestibility in all diets, before the values stabilised. Notably the variance within the protein digestibility data was the lowest of each of the three digestibility parameters. What variance there was within the protein digestibility values also minimised after two days (Figure 3). Energy digestibility values were variable over time and also took two to four days till the trend in the digestibility value stabilised. Similar to protein digestibility the variance within the energy digestibility values was also nominal and this too diminished within two to four days.

Discussion

The key foci of this study were methodological, in that the study sought to define the effects of faecal collection method and also acclimation time to diets, on the digestibility values determined in barramundi. Although studies have been performed comparing the determination of whole diet digestibilities based on faeces collected using either settlement or stripping techniques in salmonids (Windell et al., 1978; Weatherup & McCracken, 1998; Vandenberg & de la Noue, 2001; Glencross et al., 2005), this is the first study to compare the influence of these faecal collection methods with barramundi. Additionally, the study also examines the variation in digestibility over time to establish what is the best acclimation time to diets prior to faecal collection. Similar such data from other species could not be found.

Faecal collection method influences

There has been much debate on the positives and negatives associated with either faecal collection method used in digestibility studies (reviewed by Glencross et al., 2007). However, it is widely acknowledged that the two faecal collection methods do result in different diet digestibility value determinations (Windell et al., 1978; Weatherup & McCracken, 1998; Vandenberg & de la Noue, 2001; Glencross et al., 2005). These differences imply that there are compositional differences in the faeces collected which immediately have connotations on the use of each faecal collection method. Despite being more laborious and costly to collect, the data produced from faeces collected using the stripping method was more conservative than the data produced from faeces collected using the settlement method. This factor alone means that when provided with the option to use either data set the rational decision is to use the data from the stripping method because of this conservatism.

It was noted in the earlier work of Glencross et al. (2005) that the greatest differences between the nutrient digestibility assessments from the two faecal collection methods were those ingredients with higher levels of carbohydrates. A similar result was also observed in the present study with a greater number of significant differences in the digestibility of the Starch diet than either the Basal or MKM diets. It is likely that this is due to high levels of carbohydrates in the faeces decreasing faecal integrity and as such increases the dissolution of the faecal matter collected using settlement techniques.

Temporal variation in digestibility values

One of the key elements of this study was to determine the time period over which the fish should be fed a diet before faecal collection is initiated. Unfortunately there was little literature with which to compare our data in this part of the study. Therefore, in assessing this question the key parameter was considered to be the level of variability (as noted by the magnitude of the standard error) in the data collected and also how the data at any time point compares to that data obtained at

the longest acclimation time point. This was based on the assumption that by this time point the fish would have acclimated to the diet. The different digestibility parameters (dry matter, protein, energy) were also subtly different in how they responded over time with respect to the variability and also how they fared compared to the digestibility values from day 12 of the study. Fish fed the MKM diet took the longest to acclimate to it and there was a higher level of data variance within the dry matter digestibilities determined from that diet even up to day 10. However the protein and energy digestibility parameters for that diet showed little variance and were relatively consistent from day four onwards based on Figure 3.

An important observation in this study though is the level of variability seen of the data from the Basal diet. As indicated in the methods, the fish were fed this diet for one week before any faecal collection commenced, yet on day one of faecal collection a decline in dry matter digestibility was observed relative to the longer-term mean (Figure 3). In fact throughout the two week study period there was an inconsistency in the digestibility values determined for dry matter from this diet (and the other two) which perhaps indicates that some variation in digestibility might be a natural feature independent of acclimation time.

Conclusions

The two faecal collection methods used in this study are the two main methods used by fish nutritionists worldwide and this study provides a good estimate of how well each method compares when used with barramundi. The faecal stripping collection method is the more conservative of the two assessments used in this study and therefore is the one we recommend for use with this species.

When assessing the variability in digestibility over time, it was observed that in the first three days after a new diet is introduced, that the digestibility data obtained using the faecal settlement methods, was particularly variable. After this time this variability diminished and values became more uniform. We therefore recommend at least four days acclimation to new diets for barramundi before any faeces are collected for digestibility studies.

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374 Table 1. Formulations and composition diets (all values are g kg^{-1} DM unless otherwise
375 indicated) of the experimental diets
376

	Basal Diet	Starch Diet	Lupin Diet
Fishmeal	640	448	448
Fish oil [a]	100	70	70
Cellulose	124	86.8	86.8
Wheat gluten	130	91	91
Pregelled Starch	-	300	-
L. angustifolius kernel meal	-	-	300
Vitamin and mineral premix*	5	3.5	3.5
Yttrium oxide [b]	1	0.7	0.7
Dry matter	959	924	960
Protein	546	396	502
Lipid	129	85	108
Ash	106	75	82
Gross energy (MJ kg^{-1} DM)	22.0	21.0	21.0

377 * Vitamin and mineral premix includes (IU/kg or g/kg of premix): Vitamin A,
378 2.5MIU; Vitamin D3, 0.25 MIU; Vitamin E, 16.7 g; Vitamin K,3, 1.7 g; Vitamin B1,
379 2.5 g; Vitamin B2, 4.2 g; Vitamin B3, 25 g; Vitamin B5, 8.3; Vitamin B6, 2.0 g;
380 Vitamin B9, 0.8; Vitamin B12, 0.005 g; Biotin, 0.17 g; Vitamin C, 75 g; Choline,
381 166.7 g; Inositol, 58.3 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g;
382 Magnesium, 16.6 g; Manganese, 15.0 g; Zinc, 25.0 g. [a] Sourced from Skretting
383 Australia, Cambridge, TAS, Australia. [b] Sourced from SIGMA, St Louis, Missouri,
384 United States.
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386
387

388 Table 2. Chemical characterisation of the key raw materials used in this study. All values are g
389 kg^{-1} DM unless otherwise detailed.
390

Nutrient	[a]Fishmeal	[d]Lupin meal	[e]Gluten	[f]Cellulose	[e]Starch
Dry matter (g/kg)	907	902	924	927	907
Protein	744	383	710	7	10
Total lipid	75	54	46	1	1
Ash	162	34	8	2	3
Carbohydrates	19	530	236	991	986
Gross Energy (MJ/kg DM)	20.9	20.6	22.9	17.0	17.1
Alanine	47	13	20	0	0
Arginine	42	44	27	0	0
Aspartate	70	41	27	0	0
Cysteine	8	8	22	0	0
Glutamate	93	87	289	0	0
Glycine	43	16	26	0	0
Histidine	23	7	12	0	0
Isoleucine	31	16	28	0	0
Leucine	56	27	54	0	0
Lysine	55	14	10	0	0
Methionine	24	3	12	0	0
Phenylalanine	30	16	41	0	0
Proline	36	22	84	0	0
Serine	30	22	40	0	0
Taurine	7	0	0	0	0
Threonine	32	15	22	0	0
Tyrosine	24	16	28	0	0
Valine	36	15	29	0	0

391
392 Ingredient origins are as follows: [a] Fishmeal (Anchovetta meal of Peruvian origin): Ridley Aquafeeds, Narangba, QLD, Australia. [d]
393 *L. angustifolius* cv. Myallie Kernel Meal: Coorow Seed Cleaners, Coorow, WA, Australia. [e] Wheat gluten and prelatinised wheat
394 starch :Manildra, , Auburn, NSW, Australia. [f] Sourced from SIGMA, St Louis, Missouri, United States.
395

396 Table 3. Univariate MANOVA analysis with fixed effects of faecal collection method, diet
397 and method (M) x diet (D)
398

Variate	Parameter	Sum of Squares	Degrees of Freedom	Mean Square	F value	P value
Method	Dry matter	0.017	1	0.017	12.48	0.002
Diet	Dry matter	0.029	2	0.015	10.51	< 0.001
M x D	Dry matter	0.003	2	0.002	1.07	0.363
Method	Protein	0.003	1	0.003	5.83	0.027
Diet	Protein	0.001	2	0.001	1.55	0.238
M x D	Protein	0.000	2	0.000	0.19	0.830
Method	Energy	0.004	1	0.004	13.84	0.002
Diet	Energy	0.025	2	0.013	41.66	< 0.001
M x D	Energy	0.000	2	0.000	0.45	0.647

399
400

401 Table 4. Digestibility (%) specifications of diets as determined using either stripping or
402 settlement faecal collection methods. Data are mean with pooled SEM. Values within a row
403 (a,b) or between collection methods (x,y) with a different superscript are significantly
404 different ($P<0.05$).
405
406 | Nutrient | Basal | Starch | MKM | Pooled SEM |
407 |---|---|---|---|---|
408 | *Stripping* | | | | |
409 | Dry matter | $66.7^{ab, x}$ | $69.8^{a,x}$ | $59.3^{b,x}$ | 1.60 |
410 | Protein | $92.6^{a,x}$ | $91.2^{a,x}$ | $92.7^{a,x}$ | 0.77 |
411 | Energy | $82.7^{a,x}$ | $80.5^{b,x}$ | $74.5^{c,x}$ | 1.20 |
412 | *Settlement* | | | | |
413 | Dry matter | $62.3^{a,x}$ | $61.3^{ab,y}$ | $56.0^{b,x}$ | 1.35 |
414 | Protein | $94.1^{a,x}$ | $93.3^{a,x}$ | $95.5^{a,x}$ | 0.43 |
415 | Energy | $85.3^{a,y}$ | $82.3^{b,y}$ | $78.0^{c,y}$ | 0.94 |
416
417 MKM : Lupin kernel meal cv. Myallie.
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420
421
422

423 Table 5. Univariate MANOVA analysis with fixed effects of faecal collection time (T), diet
424 (D) and time x diet
425

Variate	Parameter	Sum of Squares	Degrees of Freedom	Mean Square	F value	P value
Diet	Dry matter	0.114	2	0.057	8.0	0.001
Time	Dry matter	0.081	7	0.012	1.6	0.148
D x T	Dry matter	0.094	14	0.007	0.9	0.517
Diet	Protein	0.005	2	0.003	6.4	0.003
Time	Protein	0.005	7	0.001	1.8	0.102
D x T	Protein	0.004	14	0.000	0.8	0.700
Diet	Energy	0.085	2	0.043	59.5	< 0.001
Time	Energy	0.006	7	0.001	1.1	0.346
D x T	Energy	0.048	14	0.003	4.8	< 0.001

426
427

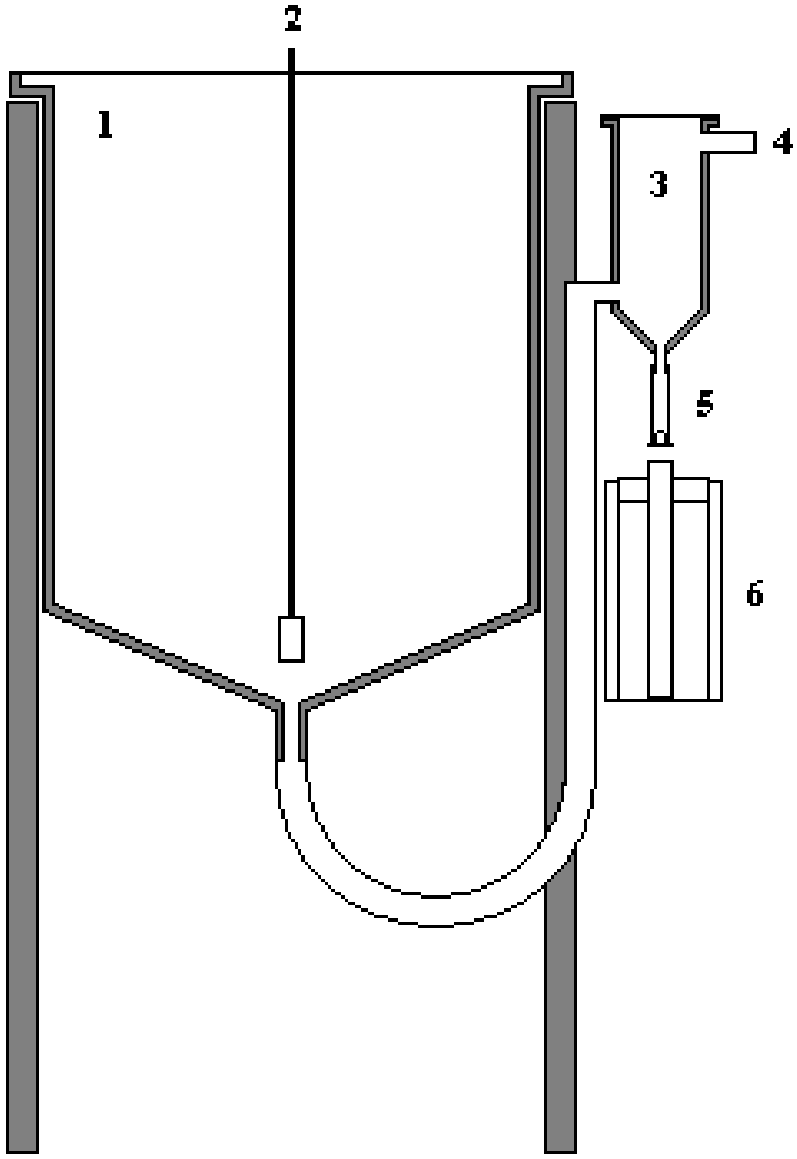


Figure 1. Design of aquaria system used to undertake the experiments from which faeces were collected by both settlement and stripping methods. Features are; 1. Conical Tank, 2. Air supply, 3. Swirl separator, 4. Waste water, 5. Silicon rubber collection tube, 6. Chiller jacket.

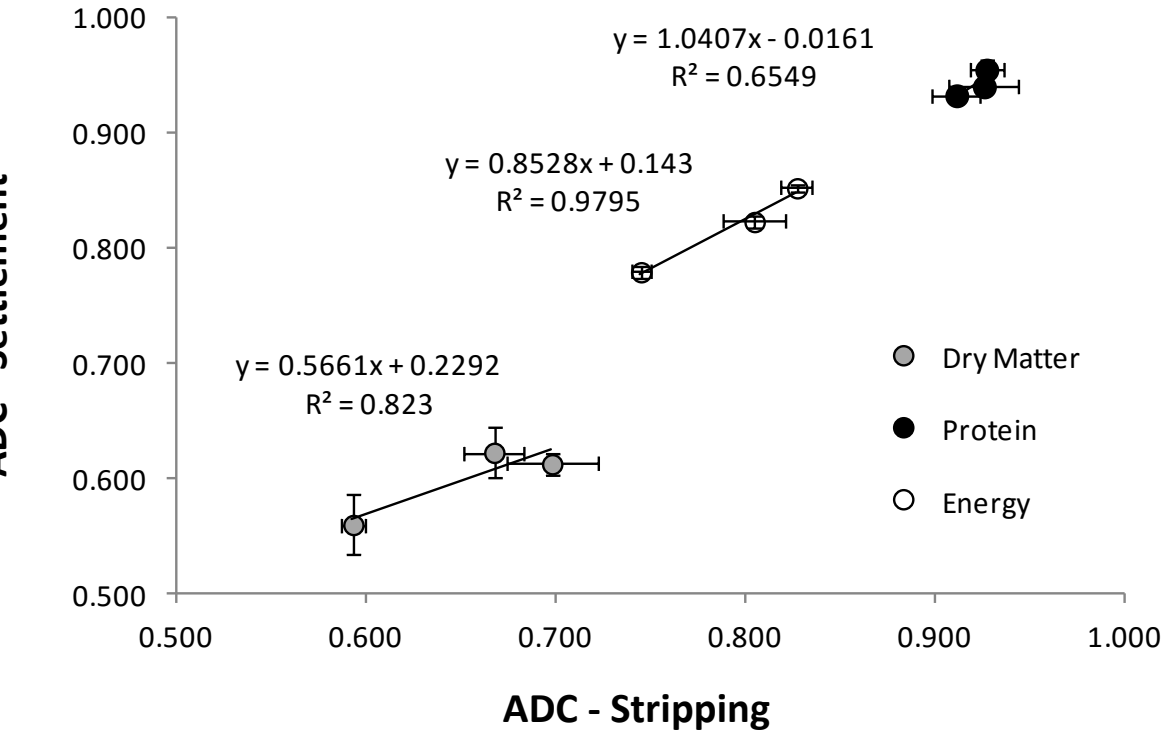
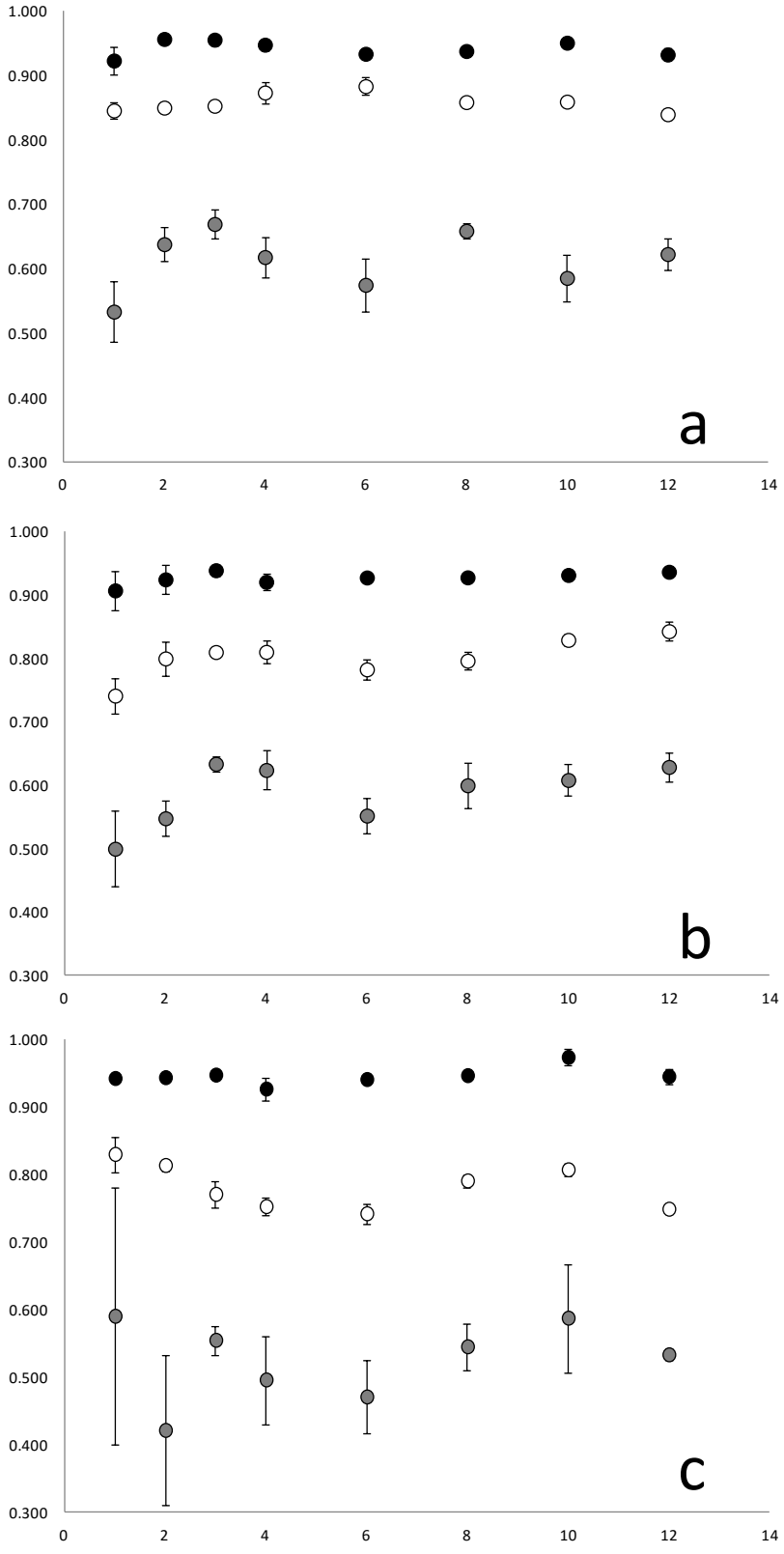


Figure 2. Correlations between apparent digestibility coefficient (ADC) values from each of the different faecal collection methods.



Figures 3a-c Temporal variation in digestibility values determined for energy (○), protein (●) and dry matter (●) for each diet (basal : a, starch : b, MKM : c) over a 13 day period.