Dietary Protein Requirement and Protein-Nitrogen Flux in the Greenback Flounder, *Rhombosolea tapirina* Günther 1862

by

Anant S. Bharadwaj

M.S. (Oceanography)
University of Rhode Island

A thesis submitted in fulfilment of the
requirements for the degree of
Master of Applied Science in
Aquaculture (by research)

Department of Aquaculture
University of Tasmania
Launceston, Tasmania

August 1997

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ACKNOWLEDGEMENTS

I thank my supervisor, Dr. Chris Carter, for his valuable advice and encouragement throughout this study. I am grateful for his thorough reviews of my writing. I also would like to thank him for introducing me to many interesting aspects of protein and energy nutrition.

The fish for this study were provided by DPIF, Taroona and I am thankful to Lance Searle, Polly Butler and Debbie Gardner for their generosity.

Brad Crear, Phil "Shelvo" Shelverton, and Greg Seeto must be thanked for their help in transporting fish from Taroona to the Key Centre.I am grateful to Brad Crear, Phil Shelverton and Rhys Hauler for baby sitting my fish on occasion. I would also like to thank Brad for sharing his modification of the ammonia analysis with me and for his assistance with trial runs of the total nitrogen analysis. Mark Johnston, Sebastian Horbushko and Rhys Hauler were of great help in the time consuming and noisome occupation of feed preparation.

I would like to express my gratitude to Dr. Greg Maguire for the use of his facilities.

I would like to thank Mark Hilder, Detlef Planko, Matt Willis, Alex Sobolewski,Craig Thomas,Greg Kent, Paul Cassidy and Doug McKinley for lending me equipment and for sharing their expertise at various times. Thanks to Conor Smith for an unlimited supply of pure distilled water.

Many thanks to Trupti, Nik and the rest of my family who supported me in various ways. Thanks to Lohitsa Inc. for making their office and its facilities available to me during the writing of this thesis.

Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any institute, college or university, and to the best of my knowledge and belief, it contains no material previously published or written by any other person, except where due reference is made in the text of this thesis.

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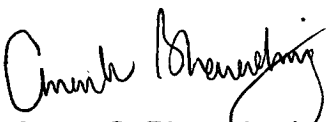


Anant S. Bharadwaj
August 1997

ABSTRACT

The overall objectives of this study were to estimate protein requirement of and to study nitrogen flux in juvenile greenback flounder, *Rhombosolea tapirina* Günther 1862.

Six isoenergetic diets (~ 17.5MJ Kg^{-1}) containing graded levels of protein ranging from 30-55% (19.24 - 34.70 gDP MJ^{-1} DE) were fed to juvenile (0.45 g) greenback flounder with the aim of estimating its dietary protein requirement. Food consumption decreased with increasing levels of protein in the diet while protein intake increased. Fish feeding on the 45% and 50% protein diets had the highest percent increases in body weight (%BWI), specific growth rates (SGR), productive protein values(PPV) and protein retention efficiencies (PRE). A second degree polynomial (quadratic) model of the form $y = a + bx + cx^2$ was fitted to dose - response data (SGR, % BWI, PPV and PRE). The dietary protein concentration required for optimal growth ranged between 44-48% and the optimal digestible protein to digestible energy ratio was estimated to be 28.14-30.41 g DP MJ^{-1} DE. The daily protein requirement at the maximum specific growth rate of 2.01 % day^{-1} was calculated to be 5.84 g kg^{-1} day^{-1}.

Endogenous and exogenous nitrogen excretion rates of juvenile(12g) greenback flounder were estimated at 16°C and nitrogen budgets constructed. Fish were fasted for 48 hours to determine endogenous

rates and fish were fed (on a diet containing approx. 46% protein) 1% body weight once a day (Treatment A), 0.5% body weight once a day (Treatment B) and 0.5% body weight twice a day (Treatment C) to determine the effect of ration size and feeding frequency on nitrogen excretion rates. The mean endogenous excretion rate of total nitrogen(TN) was found to be 23.36 mg N kg^{-1} day^{-1} with total ammonia nitrogen (TAN) and urea nitrogen (UN) constituting 82.5 and 10.4% of total nitrogen respectively. The mean exogenous total excretion rates were 402.52 mg N kg^{-1} day^{-1}, 236.60 mg N kg^{-1} day^{-1} and 398.34 mg N kg^{-1} day^{-1} for treatments A, B and C respectively. TAN and UN accounted for 83-85% and 8-9% of total nitrogen excreted in all treatments. Fish from treatment B excreted 64.48% and retained 30.52% of nitrogen consumed, while fish from treatments A and C excreted 54% and retained 40% of consumed nitrogen. Excretion rates, retention rates and percentages of consumed nitrogen excreted and retained were significantly ($p<0.05$) affected by ration size but not by frequency of feeding.

CHAPTER 1
GENERAL INTRODUCTION

1. General Introduction

Fish require protein for both maintenance and growth (Wilson and Halver 1986; Wilson 1989; NRC 1993). Proteins are an important constituent of muscle tissue, internal organs, body fluids, nervous tissue and skin (Wilson and Halver 1986) and it is therefore important that adequate quantities are supplied in the diets of fish. Proteins are used in the repair of tissue, in the formation of haemoglobin, hormones, enzymes, antibodies and other important biological compounds. Its constituent amino acids are also catabolized and used as an energy source (Wilson and Halver 1986). When protein is supplied in inadequate quantities it results in poor growth and disease while an excessive supply results in poor utilization and higher faecal and metabolic waste (NRC 1993). Therefore, a knowledge of the protein requirement of a species is necessary for the formulation of diets that promote optimal growth and reduce water pollution.

Protein requirement is defined either as the requirement for growth or the requirement for maintenance (Bowen 1987). Protein requirement for growth is generally understood as the minimum quantity of protein required to sustain maximum growth (Tacon and Cowey 1985; NRC 1993; Jobling 1994). Protein requirement is expressed in relative terms (Bowen 1987) as percentage protein in the total dry diet (Cowey *et al.* 1972; Santiago and Reyes 1991; Lochmann and Philips 1992;

Serrano *et al.* 1992;Chen and Tsai 1994; Gurure *et al.* 1995), ratio of protein to energy or digestible protein to digestible energy (Garling and Wilson 1976; Daniels and Robinson 1986; Gurure *et al.* 1995) and the ratio of protein energy to gross (total) energy (Jobling and Wandsvik 1983). Tacon and Cowey (1985) proposed expressing protein requirement in absolute terms (Bowen 1987) as a daily weight -specific protein intake at the maximum growth rate observed.

Maintenance protein requirement is the dietary protein input required to replace protein or nitrogen that is lost due to metabolism at zero growth conditions (Bowen 1987). It has been described as the protein or nitrogen required to maintain nitrogen equilibrium (Wilson 1989), where nitrogen losses are balanced by nitrogen intake with no gain in weight. This requirement has been determined using different methods. One method used has been the determination of endogenous nitrogen excretion (ENE) which has been estimated by measuring the excretions of fish fasted over a short term (Iwata 1970; Brett and Zala 1975; Cui and Wootton 1988; Carter and Brafield 1992) or of fish fed on nitrogen free diets that met the energy requirements of the fish (Gerking 1955 b; Savitz 1969). The endogenous excretion rate and maintenance nitrogen requirement has also been estimated by extrapolation of the relationship between nitrogen intake and nitrogen retention (Gerking 1955a; Birkett 1969; Jobling 1981a).

The quantitative dietary protein requirement of fish has been typically estimated using a dose-response method. This method involves the conducting of 9-12 week long growth trials where different groups of fish are fed diets containing graded levels of dietary protein. The level of dietary protein yielding optimal growth is estimated as the dietary protein requirement of the species (Tacon and Cowey 1985; NRC 1993; Jobling 1994). The amount of food consumed and changes in composition relative to a control group are also monitored to obtain information on protein utilization and retention efficiency. Optimal use of ingested protein is indicated by retention of protein or growth (Hepher 1988; Steffens 1989) with a higher efficiency of protein utilization indicating that protein has been used for growth and not metabolized for energy. Thus, data on growth and protein utilization are needed in nutrient requirement estimation and diet formulation.

Following ingestion and digestion, proteins are broken down into their constituent amino acids. These amino acids are either used for protein synthesis or are catabolized for energy. The breakdown of amino acids results in the formation of ammonia and other nitrogenous end products. Nitrogen is one of the major components of protein and studying the flux of protein nitrogen offers another approach to estimating protein retention and protein utilization efficiencies at various dietary protein levels and under different feeding regimes.

Protein-nitrogen flux can be described as the flux of ingested protein nitrogen through a fish and its basis is the nitrogen balance equation (Birkett 1969) that relates the quantity of nitrogen consumed to nitrogen losses *via* faeces and excretion. The difference in these quantities is the nitrogen retained for growth. A study of protein - nitrogen flux provides information on protein retention and nitrogen excretion rates. These are of importance in formulating diets and devising feeding regimes that minimize food wastage, faecal nitrogen losses and metabolic nitrogen losses while maximizing protein available for growth(Kaushik and Cowey 1991; Handy and Poxton 1993).

There is little information available on the quantitative protein requirement of flatfish species despite the fact that there are a few flatfish species being cultured presently. The turbot, *Scophthalmus maximus* and the sole, *Solea solea* are being currently cultured in Europe (Lahaye at. al. 1990; Guillaume *et al.* 1991). The culture of halibut, *Hippoglossus hippoglossus* is currently being evaluated in Norway (Tuene and Nortvedt 1995). The Japanese flounder or hirame, *Paralichthys olivaceous* is a popular fish in Japan and is a commonly cultured fish (Ikenoue 1983). The turbot and flounder, *Paralichthys* sp. are also being farmed in South America (Jory 1997). In North America, the *via*bility of commercial scale farming of winter flounder,

Pleuronectes americanus (Morgan 1994; Day 1996) and summer flounder, *Paralichthys dentatus* (Adams 1996) is being examined. Most of the available nutritional information on flatfish has been obtained from studies done on the turbot, plaice and sole (Guillaume *et al.* 1991). The dietary protein requirements are generally high with requirements typically at or exceeding 50% of the diet as seen in plaice, *Pleuronectes platessa* (Cowey *et al.* 1970; Cowey *et al.* 1972), turbot *Scophthalmus maximus* (Bromley 1980; Caceres-Martinez *et al.* 1984) and the sole, *Solea solea* (Guillaume *et al.* 1991).

In Australia, the greenback flounder is being examined as a possible candidate species for culture(Hart 1994). The greenback flounder, *Rhombosolea tapirina* Günther 1862, is a right handed flounder belonging to the family Pleuronectidae. It is native to southern Australian coastal waters (Coleman 1980; Last *et al.* 1983) and frequents areas with sandy and silty bottoms. Females and juveniles are found in shallow bays and estuaries while adult males are found in deeper offshore waters. During the spawning season, the females migrate offshore to the spawning grounds and larvae are transported back to the shallow coastal impoundments by the currents (Crawford 1984).

The flounder is carnivorous by nature and feeds mainly on small epibenthic prey such as harpacticoid copepods, harpacticoid nauplii

and gammaridean amphipods(Crawford 1984; Shaw and Jenkins 1992).The flounder does not have a well developed dentition and most of its teeth are concentrated on two pharyngeal plates at the posterior part of the mouth. The alimentary canal is long and coiled and typically measures 200-250% of the fish's body length. The alimentary canal is composed of a long foregut pouch and a long coiled intestine. The alimentary canal is characterized by the absence of a stomach, a pyloric sphincter and hepatic caecae (Grove and Campbell 1979).

The greenback flounder is a popular fish with high market acceptability in Australia and overseas. Market demand for this fish has not been fully met due to the inconsistent supply of fish from the wild fishery. It is thought that the farming of this species would alle*via*te this lack of supply and supply markets in the far east and south east Asia. Some of the features of the flounder that lend itself to culture are its high fecundity, hardy nature and tolerance to low levels of oxygen (Hart 1992). The fish has been spawned in captivity and larvae have been reared in captivity to the weaning stage (Crawford 1984; Hart 1994; Hart and Purser 1995; Hart *et al.* 1996). There is no information available on the nutritional requirements of juveniles of this species and the estimation of the quantitative dietary protein requirement was identified as a starting point.

The overall aims of this study were to estimate the quantitative dietary protein requirement and to study the flux of protein-nitrogen in juvenile greenback flounder under varying feeding conditions. The study was carried out in two parts. In the first part, juvenile, weaned fish (approx. 0.4g) were fed six different diets containing 30-55% protein. Data on weight increase, growth and protein retention were used to estimate dietary protein requirement. Data on food consumption and retention were used to model protein nitrogen flux at various dietary protein levels. Information on protein consumption and retention was also used to model the relationship between nitrogen intake and nitrogen retention from which the endogenous nitrogen excretion rate and maintenance protein requirement were estimated. In the second part excretion rates of juvenile flounder(approx. 10g) maintained under different feeding levels and frequencies were measured . The excretion measurements were used to model the effect of ration level and feeding frequency on protein-nitrogen flux.

CHAPTER 2
DIETARY PROTEIN REQUIREMENT OF JUVENILE GREENBACK FLOUNDER

2.1 Introduction

The greenback flounder, *Rhombosolea tapirina*, is the most abundant flatfish species in southern Australia and is a highly esteemed fish both in Australian and overseas markets (Hart 1991). Research on spawning, hatching and growout have indicated that the greenback flounder has good potential for commercial culture (Hart 1994). Currently, there is little information available on the nutritional requirements of this species and this study was undertaken to estimate the quantitative protein requirement of post weaned juveniles.

The protein requirements of various fish species have been determined using a dose-response method, where groups of fish are fed diets containing graded levels of protein. The dietary protein level that yields optimum growth (Tacon and Cowey 1985; NRC 1993; Jobling 1994) is recognized as the requirement. Various modeling approaches have been used to estimate protein requirement from dose response data in fish (Jobling 1994). These approaches have involved the fitting of broken line (Gurure et al. 1995; Chen and Tsai 1994), saturation kinetics (Gurure et al. 1995) and second order polynomial (quadratic) models (Teng et al. 1978; Santiago and Reyes 1991; Khan et al. 1993) to growth response data. Other studies have applied analysis of variance (Serrano et al. 1992; Lochmann and

Philips 1992; Catacutan and Coloso 1995) and covariance analysis (Jobling and Wandsvik 1983; De La Higuera et al. 1989) to growth data to estimate protein requirement.

The protein requirements for many different species of juvenile fish have been documented (Tacon and Cowey 1985; Wilson and Halver 1986; NRC 1993) . In general, the dietary protein requirement of fish has been observed to be 35-55%, with protein contributing 45-70% of the gross energy (Tacon and Cowey 1985). Flatfish such as the plaice, *Pleuronectes platessa*, turbot *Scophthalmus maximus* and sole *Solea solea* have typically shown a high protein requirement of 50% or higher for maximum growth (Cowey et al. 1970; Cowey et al. 1972; Bromley 1980; Caceres-Martinez et al. 1984; Guillaume et al. 1991). The greenback flounder *Rhombosolea tapirina* is a carnivorous fish (Shaw and Jenkins 1992) that feeds on small prey such as harpacticoid copepods and gammarid amphipods. The greenback flounder does not have a stomach, pyloric sphincter and hepatic caecae (Grove and Crawford 1979), but has a long foregut pouch followed by a long coiled intestine. It is also of interest whether or not these peculiarities in the digestive tract of this carnivorous fish affect protein requirement.

It has been suggested that estimates of protein requirement are dependent on the methodology used in their determination (Tacon

and Cowey 1985; Wilson and Halver 1986). Some of the factors that are thought to influence the outcome of studies are protein sources, non protein substitutes, feeding regimes and fish size. The experimental design and methodology used in this study were based on published guidelines for the conducting of nutritional requirement trials (Tacon and Cowey 1985; EIFAC 1994).

Six isoenergetic diets, containing graded levels of protein ranging from 30-55% , were fed to triplicate groups of similiarly sized (approx. 0.45 g) fish. A growth trial was carried out for 63 days during which time changes in body weight and composition were monitored. Growth and protein retention efficiency data were fit to a second order polynomial model to estimate the dietary protein concentration and ratio of digestible protein to digestible energy that supported maximum growth. Growth data, food consumption and protein retention at various protein levels were compared to evaluate the performance of diets.

2.2 Materials and Methods

2.2.1 Experimental fish

Juvenile greenback flounder, aged approximately 3 months were obtained from the Department of Primary Industries and Fisheries, Taroona, Tasmania. These fish were transported to the Key Centre for Research and Teaching in Aquaculture at the University of Tasmania, Launceston, Tasmania. At the Key Centre, they were stocked in circular 200 L Reln™ tanks supplied with recirculating sea water at 16°C and fed on a mixture of 1mm salmon and barramundi starter crumble by means of automatic belt feeders. The photoperiod was set to a 12:12 light:dark cycle. The fish were maintained in the stock tanks for approximately 1 month before being transferred to the experimental system.

2.2.2 Experimental System

The protein requirement trial was carried out in a recirculating sea water system. The experimental system used during the growth trial consisted of nineteen 28 L rectangular plastic tanks. The tanks were dark blue in colour and were equipped with lids to prevent the

accidental escape of fish during the trial. All the tanks were supplied sea water by means of a 25 mm PVC pipe with valves for each tank. The tanks were drained by 25 mm L-jointed PVC pipes that reached the bottom of the tanks. The openings of the outlet pipes were covered with 2mm mesh screens. The outflow pipes from various tanks emptied into a common gutter that in turn emptied into a 2000 L Reln™ tank via a Dacron solids filter and a biofilter. Filtered sea water was pumped into the system from the sump by means of a submersible pump. Water from the 2000 L sump was also pumped through a separate pipe to a protein skimmer that operated throughout the day. Flow rates were maintained at approximately 5 litres per minute. Each tank was supplied with an air stone and air valve to ensure continuous aeration of water. The temperature of the seawater was maintained at 16±0.5° C by controlling the temperature of the room. The photoperiod during the trial was set at 12L:12D by means of fluorescent lighting.

2.2.3 Diet Formulation

Six diets were formulated to be isoenergetic and to contain graded levels of protein (Table 2.1) ranging from 30% to 60% of the total weight, at 5% increments. The gross energy (GE) of these diets was calculated to be approximately 17.5 $MJKg^{-1}$(Table 2.2). In terms of

PE:GE (protein energy:gross energy) the protein energy to total energy ratios (Jobling and Wandsvik 1983) ranged from 0.41 to 0.74, signifying the amount of energy contributed by protein in relation to the gross energy of the diet. The energy values used for the computation of gross energy of diets are given in Table 2.2. The ratio of digestible protein to digestible energy ranged from 19.24 g digestible protein/MJ of digestible energy to 34.70 gDP/MJ DE (Table 2.2). The digestible energies of the diets were calculated on the basis of data (Jobling 1981b; Chin 1993) from experiments with plaice and greenback flounder. The diets were formulated using fish meal, gelatinized potato starch, fish oil, ascorbic acid, vitamin and mineral pre-mixes, feed attractants, fillers and antioxidants (Table 2.1). The ingredients were supplied by M/s Gibson Feed Industries, Cambridge, Tasmania.

Dietary ingredients, excluding fish oil and water, were mixed in an Atlas V-20 mixer for periods of 1 hour. The dry mixtures were stored in plastic bags at -20°C before further use. Fish Oil and distilled water (1:1) were added to the dry diets and kneaded for 1 hour in a mixer (Kenwood Mixmaster) and formed into large pellets for extrusion. The extrusion was carried out using a mincer attachment in the mixer. A stainless steel die (3.5 mm holes; fabricated by the Dept. of Engineering, Univ. of Tasmania, Launceston) with a protruding lip to

facilitate strand separation was used to extrude diets. The resulting strands following extrusion were gathered on aluminium foil sheets and dried at 28°C for 12 hours (Gallenkamp forced - fan oven). The dry strands were 2.5 mm in diameter and had moisture contents of approximately 4.5 %.

2.2.4 Preparation of Crumbles

The dried strands were broken down into pellets by crushing in a mortar and pestle. These pellets were stored in air tight polythene bags and containers at -20°C. Small portions of each of the diets were ground in the Kenwood Mixmaster's grinder attachment for periods of 30 seconds at a time and were sieved through a set of meshes ranging from 1.5 mm to 0.5mm. The particles retained by the 1mm screen were separated and stored in air tight containers for use in the growth trial. The smaller particles and the fines were stored in a freezer for use in proximate analysis.

2.2.5 Proximate analysis of diets

Moisture

The moisture content of diets was determined by drying diets at 70°C

for 14 hours. The temperature was set at 70°C in order to minimize the loss of the volatile lipid component in the diet. The samples were cooled in a desiccator and weighed at regular intervals until a constant weight was obtained. The samples were stored in a desiccator at room temperature before further analysis.

The moisture content in the diets were calculated as follows:

% Moisture = [(Orig. sample wt. - dry sample Wt.)/original sample wt)]*100

Ash

The ash contents of diets were estimated by ashing the dried feed samples(in a porcelain crucible) for 16 hours in a muffled furnace at 550°C . The samples were cooled in a desiccator and weighed at regular intervals until a constant weight was obtained.

The ash content was calculated as follows:

Ash % = (Weight of ashed sample/ weight of original sample) *100

Nitrogen

The nitrogen content of diets used in the study were determined using the Kjeldahl method (AOAC 1990). 1 g portions of dried diets were added to 10 ml of concentrated sulfuric acid. The digestion of samples was catalyzed with Selenium tablets (Kjeltabs® S3,5; K_2SO_4,Se). The

samples were digested for 1 hour at 420°C (Kjeltec® Digestion System 20). Following digestion the digestae were alkalized with sodium hydroxide (Caustic Soda; Hunter Products Tasmania Pty. Ltd.) and the resulting ammonia collected in a boric acid solution in which bromcresol green and methyl red indicators were dissolved (Kjeltec® 1026 distilling unit). This solution was titrated against 0.25 M hydrochloric acid back to the original pH of the boric acid. The nitrogen content was calculated by the following formula:

% N = (Titre - blank)* Molarity of HCl/0.05M * 1.4 * 100/wt. of sample in mg

The crude protein content of the sample was calculated by multiplying the nitrogen content by 6.25 (McDonald et al. 1992) which is the conversion factor for meat.

2.2.6 Growth trial

One hundred and ninety weaned, juvenile greenback flounder were selected from the stock tanks and ten fish randomly distributed to each of the nineteen experimental tanks. The experimental system had a triplicate set of tanks for each of the six treatments and the remaining tank held fish that would serve as the initial control group

for body composition measurements at the onset of the trial. The fish were allowed to acclimate in the experimental system for 14 days before the start of the growth trial. Two days before the trial fish were anaesthetized in benzocaine (20 ppm) and weighed to obtain their initial weights. On the first day of the growth trial the initial group of fish were sacrificed by anaesthetizing them in benzocaine (100ppm). These fish were rapidly frozen in liquid nitrogen and transferred to a freezer (-78°C) where they were stored before further analysis.
The trial was conducted over a duration of 63 days. Every 21 days the fish were anaesthetized in benzocaine (20 ppm) and weighed.
Feeding during the growth trial was maintained at three feeds per day and during all seven days of the week. The fish were fed to satiation and feeding was conducted at 0900 , 1300 and 1800 hrs.

Food was offered to fish manually and feeding was continued until there was no observable feeding activity on presentation of food. The uneaten food in each tank was siphoned out following each meal.
In addition to the siphoning of uneaten food following meals, tanks were siphoned three times daily to remove faeces and other debris.
Water temperature was maintained at 16°C and was monitored on a daily basis . Dissolved oxygen (>8mg L-1), total ammonia (<5 mgL-1) and salinity were measured three times a week. The water in the

experimental system was replaced 1-2 times a week depending on the quality of the water.

At the end of the trial, following their final weight estimation, fish were killed using benzocaine(100ppm) and rapidly frozen in liquid nitrogen. The fish were then stored in a freezer at -78°C before analysis.

2.2.7. Carcass composition analysis

Whole fish were dried in a freeze dryer (Dynavac) and weighed to constant weight. The moisture content of the carcasses was calculated as outlined earlier. Dried samples were ground into a fine powder in a grinder (Kenwood Mixmaster) and stored in airtight containers in a freezer (-78°C) before further analysis. The procedures for the determination of ash , nitrogen and protein are as outlined earlier.

2.2.8 Food consumption estimation

The amount of food consumed by fish in each tank was estimated once a week for all of the nine weeks of the growth trial. The amount of food

consumed by fish in each tank was estimated by the difference between the food offered and the food recovered following cessation of feeding. The food recovered from tanks was dried in an air forced oven at 28°C for 2 hours (calibrated using feed samples immersed in water for 15 min; n=60) and weighed to obtain the weight of food recovered.

The weights of food consumed at each of the three feedings per day were summed to obtain the food consumed per day by fish per tank. This was used to compute the food consumption per week by fish in each tank assuming a constant rate of feeding. The estimates of all the weeks were added to obtain the total food and protein consumed by fish over the course of the trial.

2.2.9 Calculations

The following calculations were made in order to evaluate the growth performance and protein retention efficiency at different dietary protein concentrations:

% Body weight increase (%BWI)

The percent increase in body weight (Moore et al. 1988) was

calculated as follows:

%BWI = [(final body wt. - initial body wt.)/initial body wt.]*100

The mean final and initial wet weights of each of the tanks in all treatments were calculated.

Specific Growth Rate (SGR)

Specific growth rate (% d^{-1}) on was calculated on a wet weight basis for each replicate in all treatments by the formula (Carter et al. 1994):

SGR (% d^{-1}) = $100*[(\ln W_t - \ln W_0)/t]$

where W_t and W_0 are the final wet weight after t days and the initial wet weight (mean weight of fish in a tank) respectively.

Productive Protein Value (PPV)

The productive protein value was calculated (Steffens 1981) as below:

PPV = increment of body protein(g)/protein intake(g)

Protein Retention Efficiency (PRE)

Protein retention Efficiency (on a dry weight basis) was estimated as follows (Millikin 1982):

PRE %= (Gain in dry body protein weight/dry protein consumed)*100

2.2.10 Statistical analysis

Mean weights of fish from each tank were used to compute weight gains and growth rates. Data from triplicate set of observations were used to estimate the mean for each treatment. Data from proximate analysis of fish carcasses were also processed in a similar fashion.

The mean initial weights of fish from all of the experimental tanks were analyzed by means of a one way analysis of variance to test for significant differences in initial weights between replicates and treatments. Data from body composition analysis and growth measurements were analyzed by a one way ANOVA and significant differences between treatment means were separated using Duncan's Multiple Range Test ($P<0.05$).

Model fit

A second degree polynomial (quadratic) of the form $y=a+bx+cx^2$ was fit to data on percent body weight increase, specific growth rate, productive protein value and protein retention efficiency with changes in levels of dietary protein inclusion. The same model was used to estimate dietary protein requirement in terms of a digestible protein to digestible energy ratio. The protein requirement (dose) for the

maximum response was calculated using the formula -b/2c (Gurure et al. 1995) . A non linear iterative procedure was used to fit the model to data. Statistical analyses and curve fitting were carried out using SigmaStat v2.01(Jandel Scientific Software, San Rafael, California).

2.3 Results

2.3.1 Experimental fish

The mean weight of all the fish used in the study was found to be 0.45±0.005 g. There were no significant differences ($p<0.05$) in the mean weights of fish used in the different treatments and between replicates. Mortalities occurred in the 30% (90% survival), 35% (93.33% survival) and 40% (96.66% survival) treatments. These mortalities occurred within 2-3 days after the trial began and were not attributed to a change in diet.

2.3.2 Proximate analysis of diets

The proximate analysis of diets revealed that the diets were formulated on the basis of calculations (Table 2.3). The values of protein contents were higher than the calculated values. Moisture content of the diets varied from 4.31% to 4.65% . The amount of ash present varied from 14. 48% to 16.01% and did not show a relationship to protein content.

2.3.3 Carcass composition

The moisture content of the initial control group was found to be 81.78% and was significantly higher than all the moisture contents of the experimental treatment groups($p<0.05$). The moisture content of

carcasses from the six dietary treatments ranged from 77.95% to 80.05% and were not significantly different ($p<0.05$) from one another (Table 2.4).

The ash content of the initial group was found to be 2.02% (wet weight basis) and was significantly lower than the 30% and 50% group. The ash content of the 40% group was significantly different from that of the 55% treatment. There was no significant difference between the mean ash values of other treatments (Table 2.4).

The protein content of the initial control group was found to be 11.34% and was significantly lower than the protein contents of all the six treatments. The protein content (on a wet weight basis) of fish increased as the dietary level of protein increased from 30% to 45% and decreased thereafter (Table 2.4). The 35,40,45 and 50% treatment levels did not have significantly different protein compositions, but they differed significantly from the 30% and 55% treatment groups. The 50% and 30% were not significantly different from the 55% treatment.

2.3.4 Growth

The maximum increase in body weight or percent body weight increase was observed at the 50% dietary inclusion level. It was found to be significantly different from the percent increase at 30,35 and 55%

inclusion levels (Table 2.5). The percent body weight increase of other treatments were not significantly different ($p<0.05$) from each other. An increasing trend was observed in the percent increase in body weight with a peak at 50% followed by a steep decline in response(Figure 2.1a).

The specific growth rates computed for the duration of the trial (63 days) showed a pattern similar to that seen in percent increase in body weight (Table 2.5). The specific growth rates were 1.35%d^{-1} and 1.28 %d^{-1} at 30 and 35% protein levels and increased to 1.75%d^{-1} and 1.78%d^{-1} at 40 and 45% (Figure 2.1b). The peak for specific growth rate was at 50% with the rate reaching 2.01%d^{-1} before declining to 1.62%d^{-1} at 55%. The specific growth rate at 50% was significantly higher than those at 30,35 and 55%, but not from those at 40 and 45%. There were no other significant differences in specific growth rate among other treatments.

The specific growth rates of flounder from the 30 and 35% treatment levels were significantly lower than the specific growth rates of fish from the other treatments (Table 2.6) during the first 21 days of the trial. Fish from the 50% level had the highest specific growth rate (0.89%d^{-1}) but did not differ significantly from the growth rates observed for the 45 and 50% treatment levels. There was no significant difference in the specific growth rates between various treatment levels

for day 21-42(week3 -6) and day 42-63 (week 6-9). However the specific growth rates at the 50% level were the highest except in the last third of the trial where fish in the 40% treatment level exhibited the highest growth rate.

Fish from some of the treatments showed variation in their growth rates at different stages of the trial. There were no significant differences in specific growth rates between the various sampling intervals for fish from the 30 and 35% treatments (Table 2.6). At the 40 and 45 % levels there was a significant difference in the specific growth rates between the day 1-21 and day 21-42 periods. There was no difference of statistical significance between the second and the third period. At the 50% level the first period showed a significantly higher growth than the third period. At 55% the third period showed a significant decline in specific growth rate compared to the preceding two periods.

2.3.5 Food Consumption

The amount of food consumed by fish varied inversely with the protein level in diets (Figure 2.2). The mean amount of food consumed by individual fish over the length of the trial was highest for the 30% protein diet and lowest for the 55% diet. The amount of food consumed by fish fed 30 and 35% protein diets were significantly higher than fish fed 40, 45, 50 and 55% protein diets (Table 2.7). Food consumption at

the 55% level was significantly lower than that at 40%. There was no significant difference in food consumption by fish fed the 40, 45 and 50% protein diets. Fish fed the 45,50 and 55% protein a diets also had no significant differences in food consumption.

The amount of protein consumed (wet weight) did not show a definite relationship with the level of protein in the diet (Figure 2.2). The only significant difference in the protein consumption was between the 30 and 50% treatments (Table 2.7).

2.3.6 Protein retention

Productive protein values (PPV) were highest at the 40, 45 and 50% treatment levels(Fig 2.3a). The maximum value of 37.52 occurred at the 45% treatment level and showed a decline at the 50 and 55% levels. The PPV's at the 40, 45 and 50% treatment levels were not significantly different from one another, but were significantly higher than the PPV's observed for the 30,35 and 55% diets (Table 2.8). The 30,35 and 55% diets did not produce significantly different PPV values.

Protein retention efficiency was found to be at its maximum for fish fed the 50% protein diet (Figure 2.3b). The protein retention at the 50% treatment level was significantly higher than the retention at the 30 and 35% levels (Table 2.8). The protein retention at 30% was not

found to be significantly higher than the 35%. The protein retention efficiency at 40 and 45% while significantly higher the 35% level were not significantly higher than the 30% level.

2.3.7 Estimation of protein requirement

The dietary concentration of protein that supported maximum growth in juvenile flounders in this study ranged from 44.40% to 48.11% depending on the response modelled(Table 2.9). The fit of percent body weight increase data to the second order polynomial model predicted maximum growth at 48.11% and the fit of specific growth rate data predicted maximum growth at 48.09%. The fit of protein productive value and protein retention efficiency data to the model predicted dietary protein requirement of 44.40% and 46.58%. The fit of percent body weight increase, specific growth rate, productive protein value and protein retention efficiency data to the second order polynomial (quadratic) model are shown in Figure 2.4. The model was fitted to the above data using digestible protein to digestible energy ratios as the dose and the DP/DE ratio that promoted maximum growth ranged from 28.14 g DP/ DE to 30.41 g DP/MJ DE (Table 2.9). The fit of data to the model for the estimation of digestible protein to digestible energy required for maximum growth is shown in Figure 2.5.

2.4 Discussion

The dietary protein requirement of juvenile greenback flounder as estimated from this study was found to range between 44 and 48%. The predicted protein requirement from weight gain, growth and protein utilization data were in good agreement. This supports the use of protein utilization data in addition to weight gain and growth data in the estimation of protein requirement. Protein requirement expressed as grams digestible protein per MJ digestible energy ranged from 28.14 to 30.4 g DP/MJ DE. Model fits to PRE and PPV data predicted lower protein requirements than % BWI and SGR data.

This dietary protein requirement of the flounder lies within the range of 35-55% observed for most fish and is typical of the dietary protein composition required by most carnivorous fish (DeLong *et al.* 1958; Cowey *et al.* 1972; Zeitoun *et al.* 1973; Satia 1974; Lall and Bishop 1977; Bromley 1980; Anderson *et al.* 1981; Caceres-Martinez *et al.* 1984; Gurure *et al.* 1995) The dietary protein requirement of omnivorous fish has generally been observed to be lower and in the range of 29-45% (Garling and Wilson 1976; Dabrowski 1977; Chuapoehuk 1987; Davis and Stickney 1978; Lochmann and Phillips 1994). The dietary protein requirements of the greenback flounder were similar to the plaice, *Pleuronectes platessa* (Cowey *et al.* 1972), where maximum weight gain was observed at the 50% dietary

inclusion level. In contrast maximum growth rates in the turbot, *Scophthalmus maximus* were observed at higher dietary inclusion levels of around 69-80% (Bromley 1980; Caceres-Martinez *et al.* 1984).

The optimal DP/DE ratio of juvenile greenback flounder appears to be higher than that seen in other species for which data is available. Optimum protein to energy ratios expressed in terms of mg/Kcal for various species (NRC 1993) were recalculated and expressed in terms of g/MJ so as to facilitate comparison. The optimal DP/DE ratio in channel catfish ranged from 19.12 to 23.21 g/MJ. The values for other species were recalculated as follows: red drum, 23.52 g/MJ; hybrid bass, 26.88 g/MJ; Nile tilapia, 24.72 g/MJ; common carp, 25.96 g/MJ; rainbow trout, 21.90 and 23.90 g/MJ. Data from a study on the Arctic charr (Gurure *et al.* 1995) estimated the optimal DP/DE ratio at approximately 22 g/MJ. These values are lower than the 28.14 - 30.41 g/MJ optimal DP/DE ratio observed in the greenback flounder.

The diets used in this study were formulated to be isoenergetic while varying in their protein concentration. The energy values of diets were calculated both on basis of gross and digestible energy. During formulation, changes in the ratio of protein level or protein energy to gross energy were achieved without altering the gross or digestible energy significantly. The gross or digestible energy of diets were not

experimentally determined and existing data were used in their calculation. Chin (1993) estimated protein digestibility to be around 95% in greenback flounder weighing 15 g. Since no data were available for energy digestibility, data from Jobling (1981b) on energy digestibility in plaice were used. In that study juvenile plaice were fed fish meal based diets with an energy density of approximately 17 MJ kg^{-1}, which was close to the energy density of diets used in this study. Ideally, the digestibility coefficients for energy and nutrients need to be determined for each study, but this was made difficult due to the refusal of greenback flounder to feed on chromic oxide diets. Jobling (1983) has expressed reservations on the application of digestibility coefficients from one species to another or even within a species. Factors such as species, size, diet composition, temperature and feeding regime possibly influence digestibility. Jobling (1983) however also points out that there is no conclusive trend in the literature showing the effects of these variables on digestibility.

Analysis of variance of growth data showed significant differences in the various growth responses measured and calculated. Although there were significant differences, there was not enough separation to clearly determine the optimal protein requirement. Analysis of variance and multiple comparison of means of percent increase in body weight specific growth rate, productive protein value and protein retention efficiency data predicted optimal protein requirement to lie between

40 and 50%. In general, maximum values of all the measures were found at the 50% level. The fit of data to a non linear regression model provided a more precise estimate of protein requirement.

In this study the polynomial model was used over other models based on preliminary fits of models to data. Models with plateaus such as saturation kinetics models, exponential models and broken line models suffered from a lack of fit as determined by examining residuals from model fits. The second order polynomial (quadratic) model is asymptotic and parabolic in shape and showed a greater degree of fit to data. This might lie in the nature of the data which shows a definite declining trend from 50% to 55%. The value of the growth response at 55% was generally lower than the values at 45% and 40% suggesting that the data had a peak around 50%.

Models with plateaus predict a leveling off in growth response with increase in dietary concentrations of nutrients (Robbins *et al.* 1979) and it was felt that these models would not be appropriate for data from this study. Robbins *et al.* (1979) have suggested that failure of the broken line model to fit data adequately indicate the need for a curvilinear model. They also recommend the *a priori* selection of a curvilinear model if the data show an obviously curvilinear nature.

Analysis of variance, broken line analysis, saturation kinetics models and polynomial regressions have led to similar conclusions in some protein requirement studies (Moore *et al.* 1988; Gurure *et al.* 1995). The protein requirement predicted by the broken line and saturation kinetics model were both lower than the estimate from the fit to the quadratic model in the Arctic charr, *Salvelinus alpinus* (Gurure *et al.* 1995). Zeitoun *et al.* (1976) are of the opinion that the polynomial regression is a continuous model and provides a smooth approximation of the relation between weight gain and protein intake. It also permits the use of costs and returns for economic analysis (Zeitoun *et al.* 1976).

In the designing of the growth trial, effort was made to follow guidelines outlined in the literature (Tacon and Cowey 1985; EIFAC 1994) to avoid any bias towards any particular treatment. Diets were formulated using fish meal as a protein source. Fish meal is considered to have a balanced amino acid profile that matches that of fish and is also recommended as a standard reference protein (Tacon and Cowey 1985). This study was conducted using practical diets as purified diets are thought to decrease palatability and food intake. As optimal growth is used to define dietary protein requirement it is essential that food is not a limiting factor. When the feeding level is increased, the protein requirement decreases. This has been observed in carp and rainbow trout (Ogino 1980), where the protein

requirement was halved with a doubling in feeding level. In the present study, fish were fed to satiation and were fed three times per day. Earlier studies on the greenback flounder (Chin 1993) have shown that , while there was an increase in food intake, there was no significant increase in growth rate, food utilization and digestibility between for feeding frequencies permits of 1-4 times a day. The feeding protocol employed here (2-4 feeds per day; satiation feeding) has been used in a number of other protein requirement studies (Cowey *et al.* 1972; Sabaut and Luquet 1973; Zeitoun *et al.* 1973; Anderson *et al.* 1981; Millikin 1983; Jobling and Wandsvik 1983). The effects of fish size were minimized by using fish of similar size and by testing for significant differences in mean size within replicates and treatments at the beginning of the trial.

The estimation of food consumed by fish in this trial was made difficult by the small size of the fish. The use of radiographic methods (Carter *et al.* 1994) was precluded because fish were fed crumble and there would be considerable error in estimating consumption due to the loss of glass beads during the preparation of crumble and during ingestion. The method used in this study involved feeding a known quantity of feed and recovering uneaten portions. It is probable that a degree of error was introduced by failure to recover all the uneaten food and by the recovery of some faecal material, even though great attention was paid to removal of faecal matter before feeding.

Food consumption decreased with increase in protein content of the diet. This has been observed in other studies with plaice, (Cowey *et al.* 1972), Arctic charr (Gurure *et al.* 1995), estuary grouper,(Teng *et al.* 1978) and turbot (Calcedo 1990). There was a significant difference in food consumption between fish fed 30-35% protein diets and the diets with higher protein content. The consumption of protein did not show significant variation with increase in dietary protein content, with only intake at the 30% level being significantly lower than that at the 50% level. Calcedo (1990) also observed no significant difference in protein consumption in fish fed diets containing protein ranging from 30-60%. Cowey *et al* .(1972) observed an increase in protein consumed with increase in protein level in the diet and attributed the lower food intake at higher protein levels due to their relatively higher energy contents. There is no obvious explanation as to why the flounders in this study ingested more food when fed diets with a lower protein concentration. It is probable that intake at lower protein levels was maximized to meet both energy and protein requirements.

Protein retention efficiency (PRE) and productive protein value (PPV) were used in favour of other measures such as feed efficiency, food conversion ratio and protein efficiency ratio (PER) to estimate the efficiency of protein utilization. Feed conversion ratio, feed efficiency

and protein efficiency ratio may not be completely adequate measures for evaluating dietary performance (Hepher 1988). Feed conversion ratio is the dry weight consumed for gain in wet weight and feed efficiency is the wet weight gained by dry weight of food consumed. These evaluating criteria do not take into account the contribution of fat to weight gain. PER relates weight gain to protein intake and like FCR, PER does not consider weight gain by increase in fat content. It also evaluates the protein rather than the diet itself and assumes that all the protein is used for growth. They are however useful in providing general information on food utilization and performance at different dietary protein concentrations.

In conclusion, the dietary protein concentration required for optimal growth was estimated to range between 44 and 48% of total diet weight. It was also estimated that 29 - 30 g of digestible protein per MJ of digestible energy promoted optimal growth in this size class of flounder under the prevailing experimental conditions.

Table 2.1. Composition of experimental diets fed to juvenile greenback flounder.

	Dietary Protein Content %					
	55%	50%	45%	40%	35%	30%
Ingredient %						
Fishmeal	84.6	76.9	69.2	61.5	53.9	46.2
Fishoil	5.6	9.16	9.74	10.33	10.90	11.49
Potato Starch	0	0	6.52	13.00	19.40	25.90
Bentonite	1.80	5.94	6.54	7.17	7.80	8.41
∝-Cellulose	5	5	5	5	5	5
Carboxymethyl Cellulose	2	2	2	2	2	2
Minerals [1]	0.25	0.25	0.25	0.25	0.25	0.25
Vitamins [2]	0.5	0.5	0.5	0.5	0.5	0.5
L-Ascorbic Acid [3]	0.05	0.05	0.05	0.05	0.05	0.05
Feed Stimulants [4]	0.18	0.18	0.18	0.18	0.18	0.18
Butylated Hydroxyanisole (BHA)	0.02	0.02	0.02	0.02	0.02	0.02
Total	100	100	100	100	100	100

[1] supplied to meet requirements outlined by NRC(1993)
[2] supplied to meet requirements outlined by NRC(1993)
[3] L-Ascorbic acid (Static C®) was manufactured by Roche Ltd. (Norway)
[4] Feed Stimulants consisted of equal proportions of betaine, glycine, inosine and L-alanine

Table 2.2. Calculated crude composition and energy content of experimental diets fed to juvenile greenback flounder

	Dietary Protein Content %					
	55%	50%	45%	40%	35%	30%
Composition (%.DM^{-1})						
Crude Protein	54.99	49.99	44.98	39.98	35.04	30.03
Crude Fat	12.03	15.00	14.99	15.00	14.99	15.00
Starch	0	0	6.52	13.00	19.40	25.90
Crude Fibre	7	7	7	7	7	7
Total Carbohydrate	7	7	13.52	20.00	26.40	32.90
Ash	18.72	21.32	20.38	19.47	18.58	17.65
Energy MJKg^{-1}						
Gross Energy[1] (GE)	17.71	17.70	17.64	17.57	17.50	17.45
Digestible Energy[2] (DE)	15.05	15.04	14.99	14.94	14.88	14.83
Protein Energy (PE)	13.19	11.99	10.79	9.59	8.40	7.20
g Dig. Protein MJ^{-1}DE	34.70	31.55	28.49	25.42	22.36	19.24
PE:GE	0.74	0.68	0.61	0.54	0.48	0.41

[1] Assuming energy values of 23.59 MJKg^{-1} for protein, 39.40 MJKg^{-1} for fat and 17.19 MJKg-1 for carbohydrate (NRC 1993; Jobling 1994).

[2] Assuming digestibility of protein= 95% (Chin 1993); energy digestibility = 85% (Jobling 1981b).

Table 2.3. Proximate analysis of experimental diets fed to juvenile greenback flounder (means±SE)

	Dietary Protein Content %					
	55%	50%	45%	40%	35%	30%
Moisture % (n=3)[1]	4.61±0.02	4.56±0.09	4.55±0.08	4.65±0.05	4.31±0.02	4.61±0.04
Ash % (n=3)[2]	14.48±0.38	16.01±0.57	15.73±0.60	15.73±0.05	15.85±0.04	15.16±0.01
Nitrogen %(n=3)[3]	8.93±0.12	8.20±0.05	7.32±0.13	6.73±0.13	5.82±0.15	5.15±0.05
Protein %[4]	55.78±0.77	51.26±0.32	45.79±0.84	42.07±0.86	36.39±0.93	32.23±0.32

[1] oven dried at 70°C to constant weight
[2] heated in a muffle furnace at 550°C for 16 h
[3] kjeldahl method
[4] calculated by multiplying kjeldahl N* 6.25

Table 2.4. Carcass composition of juvenile greenback flounder fed six different experimental diets. Values[1] within a column with different superscripts are significantly different($P < 0.05$)

Dietary Protein %	Carcass moisture %	Carcass protein[2] %	Carcass ash[2]
Initial	81.78±0.56	11.34±0.09	2.02±0.04
30	77.99±1.06^{a}	12.33±0.18^{c}	2.32±0.07^{ab}
35	80.05±1.11^{a}	13.33±0.13^{a}	2.04±0.05^{ab}
40	79.59±0.94^{a}	13.78±0.41^{a}	2.10±0.08^{a}
45	78.13±0.17^{a}	13.96±0.28^{a}	2.25±0.09^{ab}
50	77.95±1.00^{a}	13.10±0.21^{ab}	2.37±0.06^{ab}
55	78.91±0.45^{a}	12.14±0.17^{bc}	2.20±0.04^{b}

[1] Means ± S.E. of three replicates
[2] wet weight basis

Table 2.5. Growth responses of juvenile greenback flounder to six experimental diets after 63 days. Values[1] within a column with different superscripts are significantly different.

Dietary Protein %	% BWI[2]	SGR[3] %d-1
30	145.57±32.82^{b}	1.35±0.21^{b}
35	131.86±21.71^{b}	1.28±0.14^{b}
40	213.56±20.34^{ab}	1.75±0.10^{ab}
45	219.65±17.66^{ab}	1.78±0.08^{ab}
50	273.64±31.57^{a}	2.01±0.13^{a}
55	189.19±22.20^{b}	1.62±0.12^{b}

[1] Values are means ± SE for three replicates
[2] % BWI, Percent Body weight increase
[3] SGR, Specific growth rate

Table 2.6. Specific growth rates of juvenile greenback flounder at 21 day intervals . Values [1] within a column(lower case letters) and a row(capitals) with different superscripts are significantly different.

Protein Level %	Day 1-21	Day 21-42	Day 42-63
30	0.47 ± 0.08^{bcA}	0.47 ± 0.08^{aA}	0.45 ± 0.06^{aA}
35	0.47 ± 0.06^{cA}	0.51 ± 0.09^{aA}	0.33 ± 0.02^{aA}
40	0.81 ± 0.03^{acA}	0.48 ± 0.02^{aB}	0.50 ± 0.07^{aB}
45	0.88 ± 0.07^{aA}	0.54 ± 0.05^{aB}	0.41 ± 0.02^{aB}
50	0.89 ± 0.10^{aA}	0.73 ± 0.06^{aAB}	0.45 ± 0.04^{aB}
55	0.66 ± 0.11^{abcA}	0.67 ± 0.15^{aA}	0.34 ± 0.09^{aB}

[1] Values are means ± SE of three replicates

Table 2.7. Food consumption by juvenile greenback flounder fed diets containing different protein levels. Values[1] within a column with different superscripts are significantly different .

Dietary Protein %	Food Consumption g	Protein Consumption g
30	1.15 ± 0.05^{b}	0.37 ± 0.02^{b}
35	1.01 ± 0.05^{b}	0.40 ± 0.02^{ab}
40	0.99 ± 0.04^{c}	0.42 ± 0.02^{ab}
45	0.86 ± 0.02^{ac}	0.39 ± 0.01^{ab}
50	0.90 ± 0.02^{ac}	0.46 ± 0.01^{a}
55	0.78 ± 0.04^{a}	0.43 ± 0.02^{ab}

[1]Values are means± SE of three replicates; measurements are for individual fish over the duration of the growth trial (63 days) and are on a wet weight basis.

Table 2.8. Protein retention in juvenile greenback flounder fed diets containing varying levels of protein. Values within a column with different superscripts are significantly different ($P < 0.05$).

Dietary Protein %	PPV[1] %	PRE[2] %
30	23.58 ± 4.61^{b}	29.24 ± 5.38^{bc}
35	22.01 ± 3.10^{b}	20.21 ± 2.98^{c}
40	35.40 ± 2.90^{a}	35.48 ± 2.93^{ab}
45	37.52 ± 0.82^{a}	38.09 ± 0.92^{ab}
50	36.59 ± 2.68^{a}	42.45 ± 2.37^{a}
55	24.72 ± 2.13^{b}	28.87 ± 2.37^{abc}

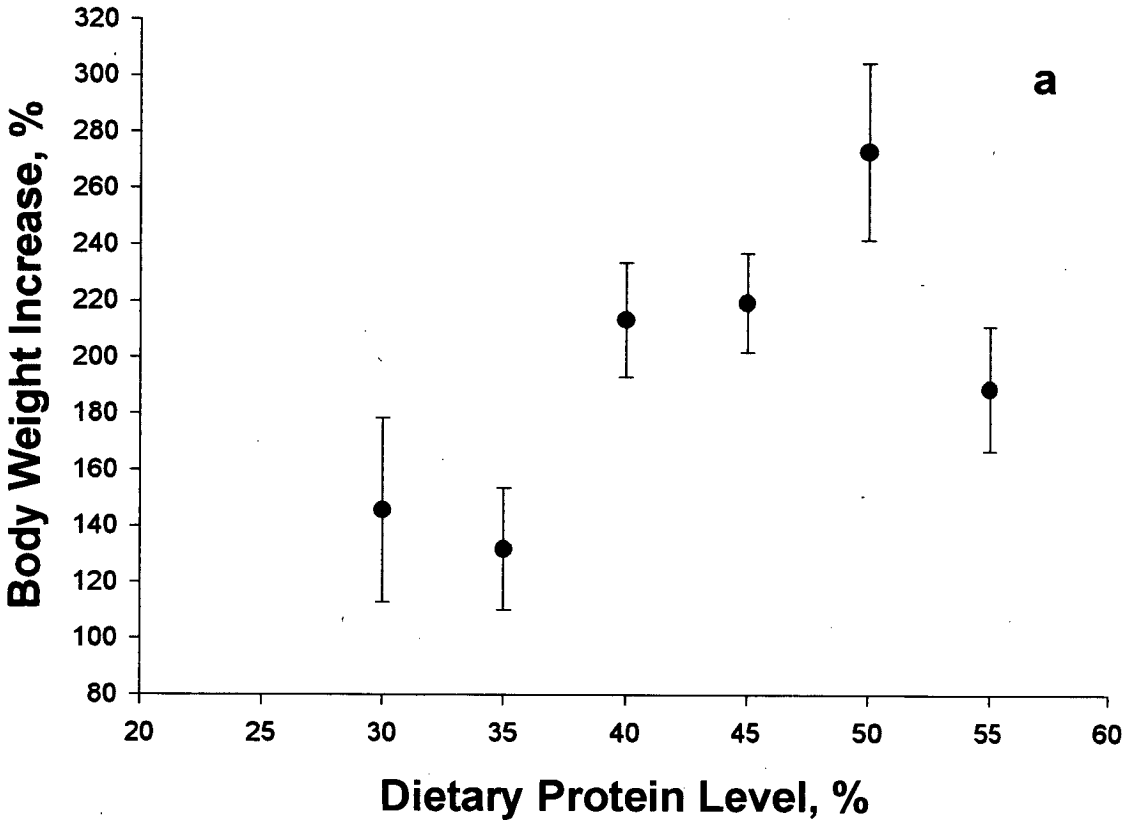
[1] PPV, productive protein value (on a wet weight basis)
[2] PRE, protein retention efficiency(on a dry weight basis)

Table 2.9. Protein requirement estimates for juvenile greenback flounder obtained by fitting a second order polynomial (quadratic) model to growth response and protein retention efficiency data.

Response	Model Equation	Protein Requirement Estimates	
		% of diet	g DP MJ^{-1} DE[5]
%BWI[1]	$Y = a + bx + cx^2$	48.09%	30.41 g MJ^{-1}
SGR[2]		48.11%	30.37 g MJ^{-1}
PPV[3]		44.40%	28.14 g MJ^{-1}
PRE[4]		46.58%	29.51 g MJ^{-1}

[1] % BWI , percent increase in body weight
[2] SGR, specific growth rate, % d^{-1}
[3] PPV, productive protein value, %
[4] PRE, protein retention efficiency, %
[5] g digestible protein per MJ of digestible energy

Figure 2.1. Growth response of juvenile greenback flounder to varying levels of dietary protein (a) percent body weight gain, %BWI and (b) Specific growth rate, SGR % d^{-1}. Values are mean values± S.E.


Body Weight Increase, %
320
300
280
260
240
220
200
180
160
140
120
100
80
a
20
25
30
35
40
45
50
55
60
Dietary Protein Level, %

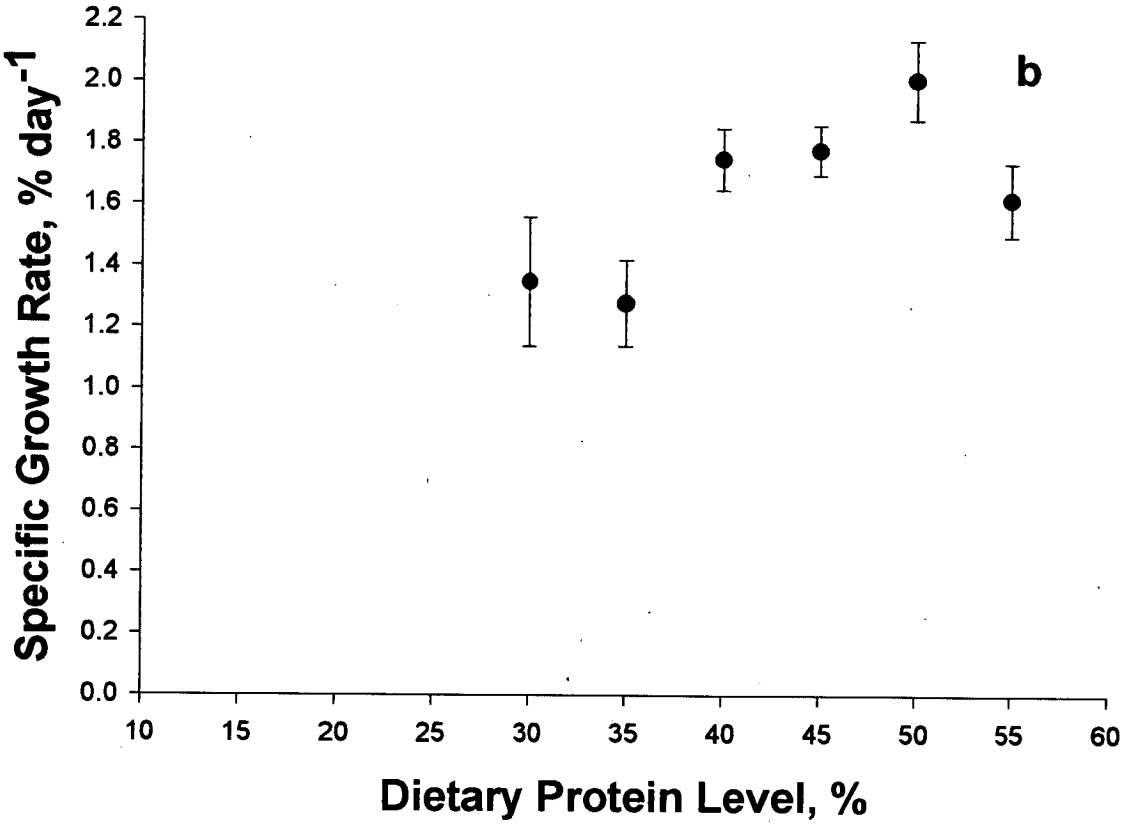

Specific Growth Rate, % day-1
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
b
10
15
20
25
30
35
40
45
50
55
60
Dietary Protein Level, %

Figure 2.2. Food and protein consumption by juvenile greenback flounder fed diets containing different protein levels. Values are mean values± S.E.

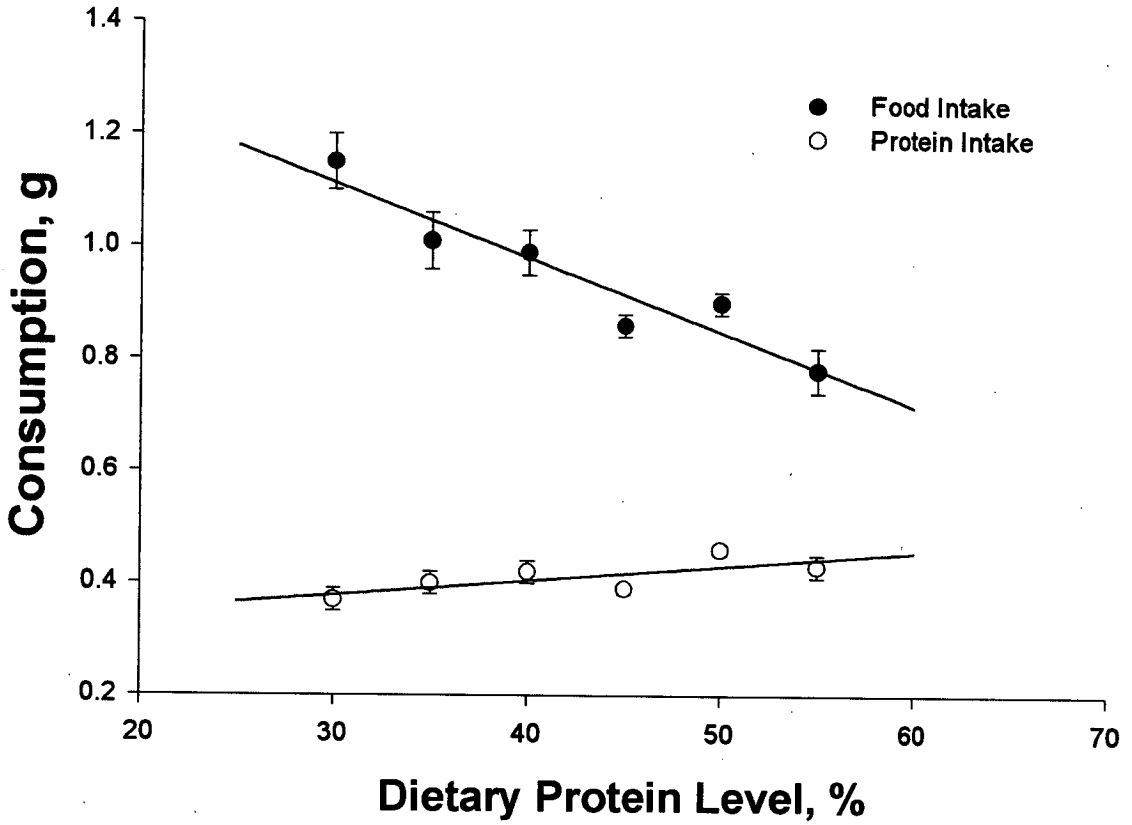
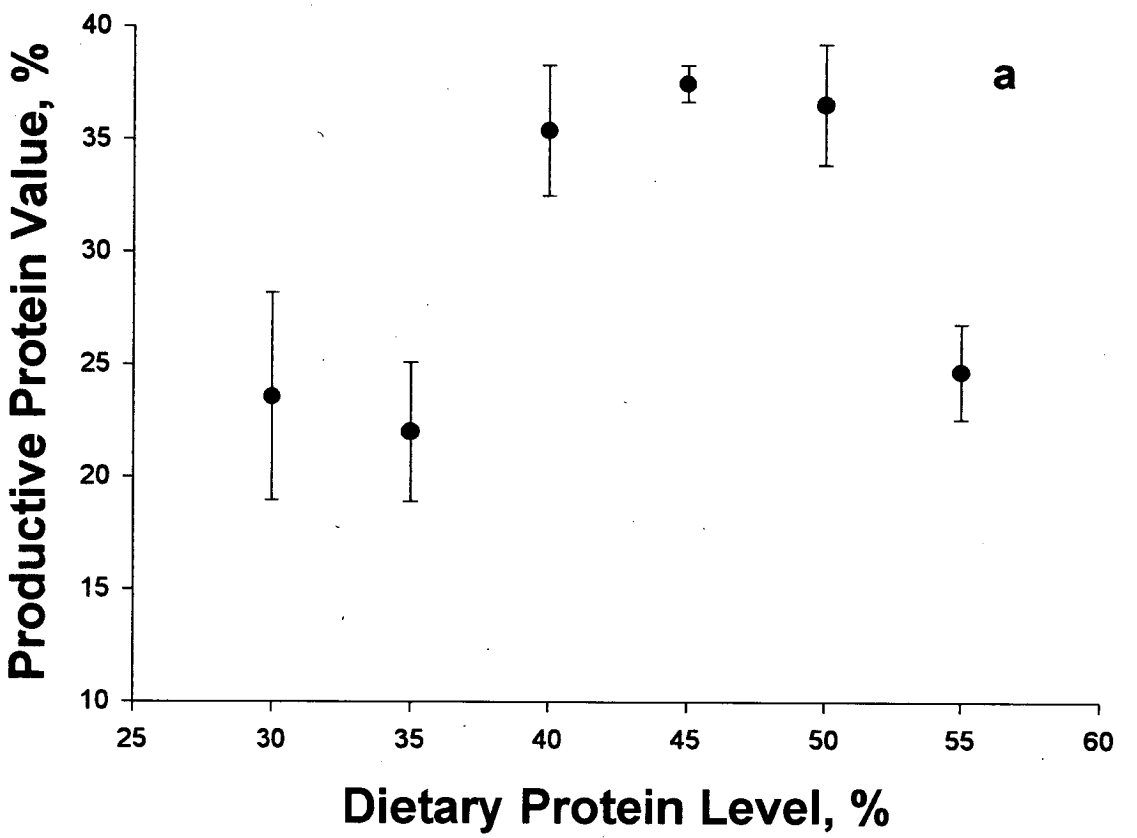

Food Intake
Protein Intake
Consumption, g
1.4
1.2
1.0
0.8
0.6
0.4
0.2
20
30
40
50
60
70
Dietary Protein Level, %

Figure 2.3. Protein retention in juvenile greenback flounder fed diets containing varying levels of protein (a) Productive protein value, PPV (b) Protein retention efficiency, PRE.


Productive Protein Value, %
40
35
30
25
20
15
10
25
30
35
40
45
50
55
60
Dietary Protein Level, %
a

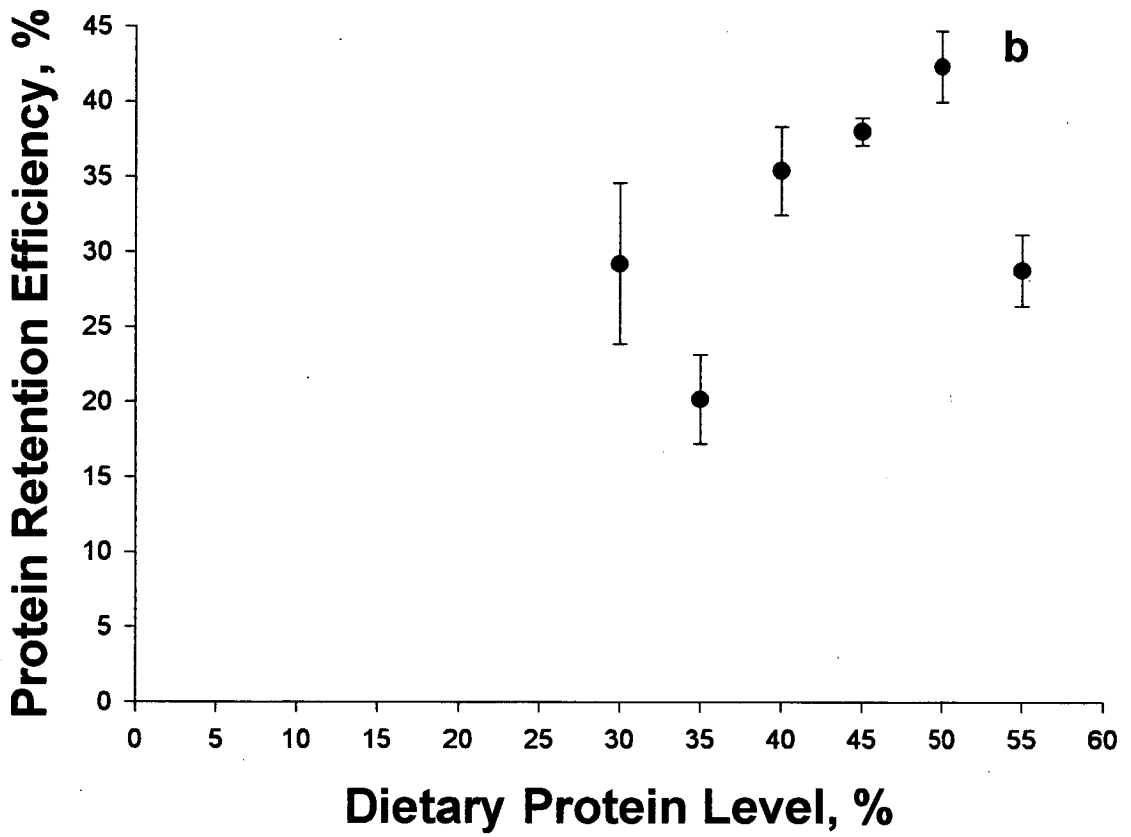
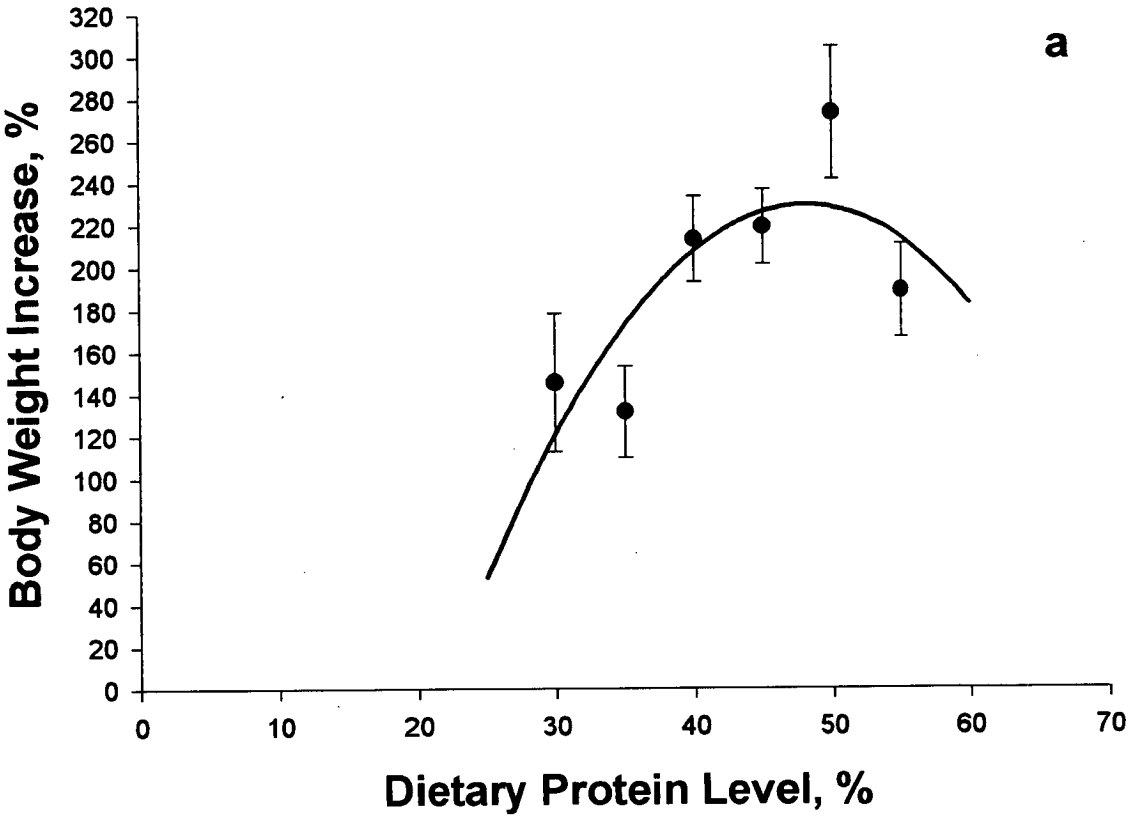
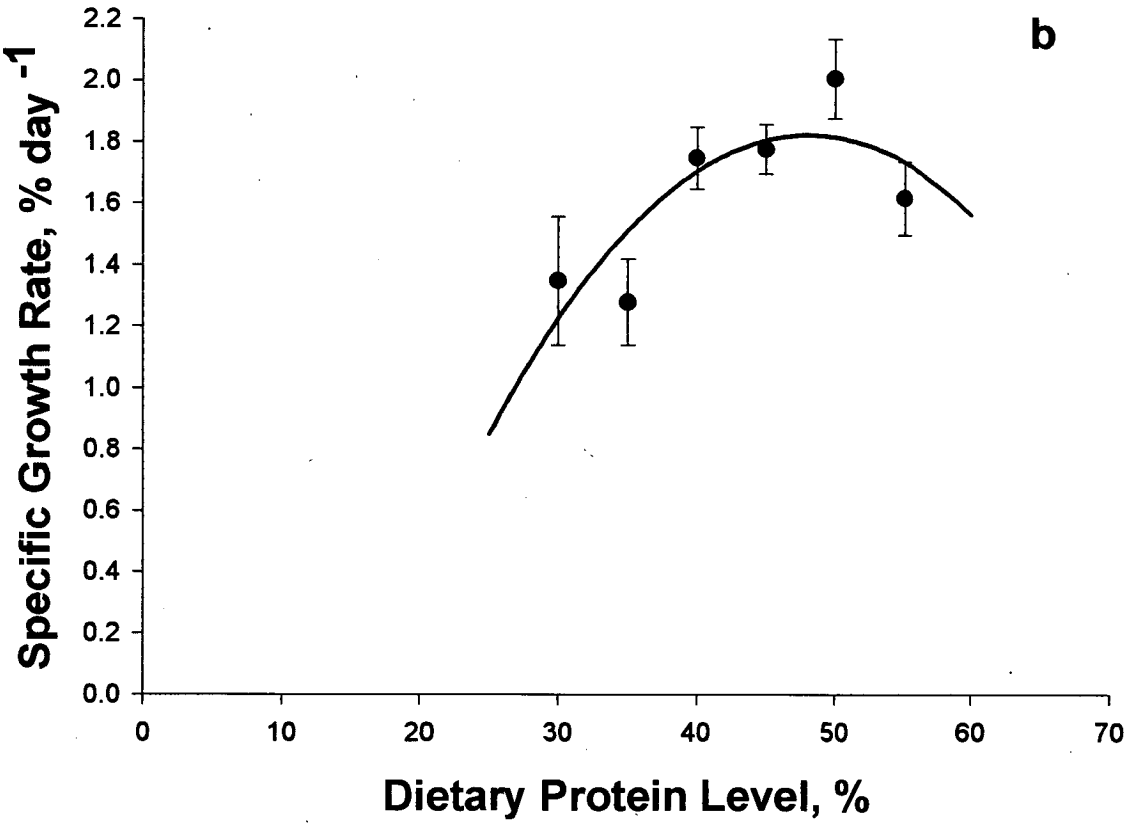
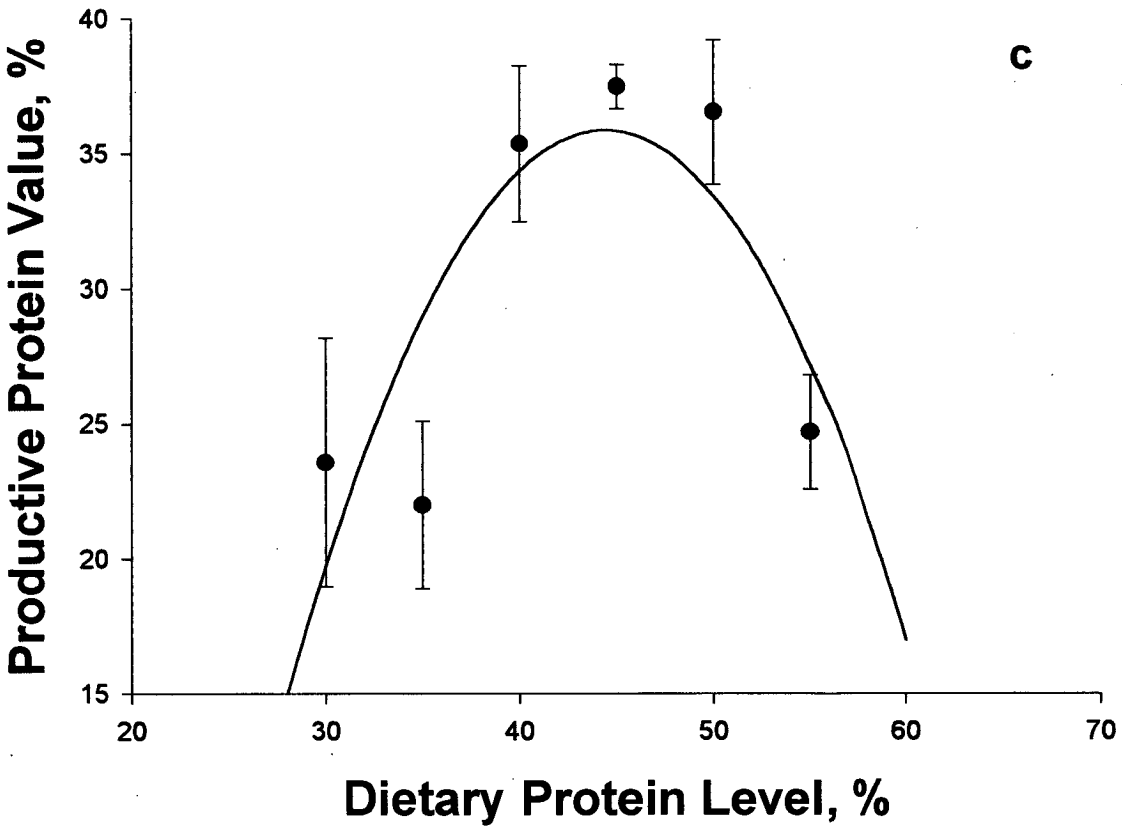

Protein Retention Efficiency, %
45
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
35
40
45
50
55
60
b
Dietary Protein Level, %

Figure 2.4. Fit of (a) % increase in body weight (b) specific growth rate (c) Productive protein value and (d) protein retention efficiency data to a second order polynomial (quadratic) model for estimation of protein requirement in terms of dietary percentage. Model details are given in the text. Values are mean values ± S.E.


a
Body Weight Increase, %
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
Dietary Protein Level, %


b
Specific Growth Rate, % day -1
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
Dietary Protein Level, %


c
Productive Protein Value, %
40
35
30
25
20
15
20
30
40
50
60
70
Dietary Protein Level, %

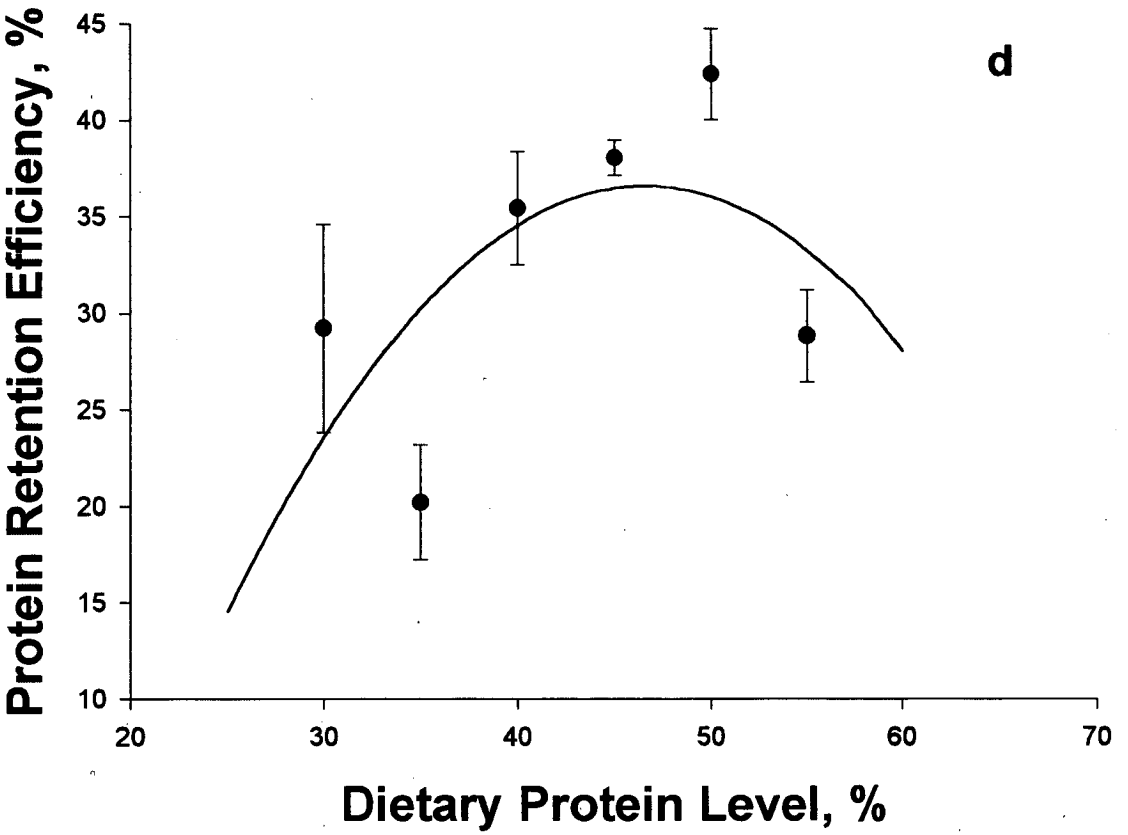
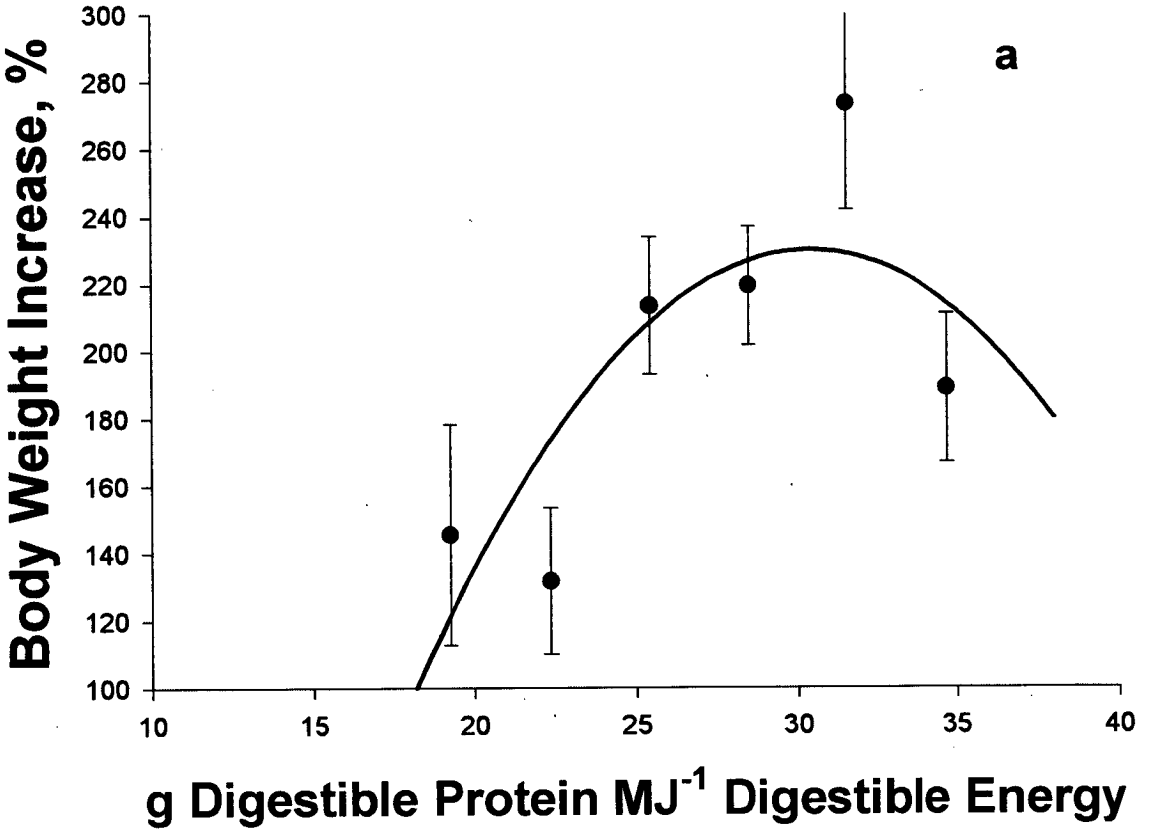
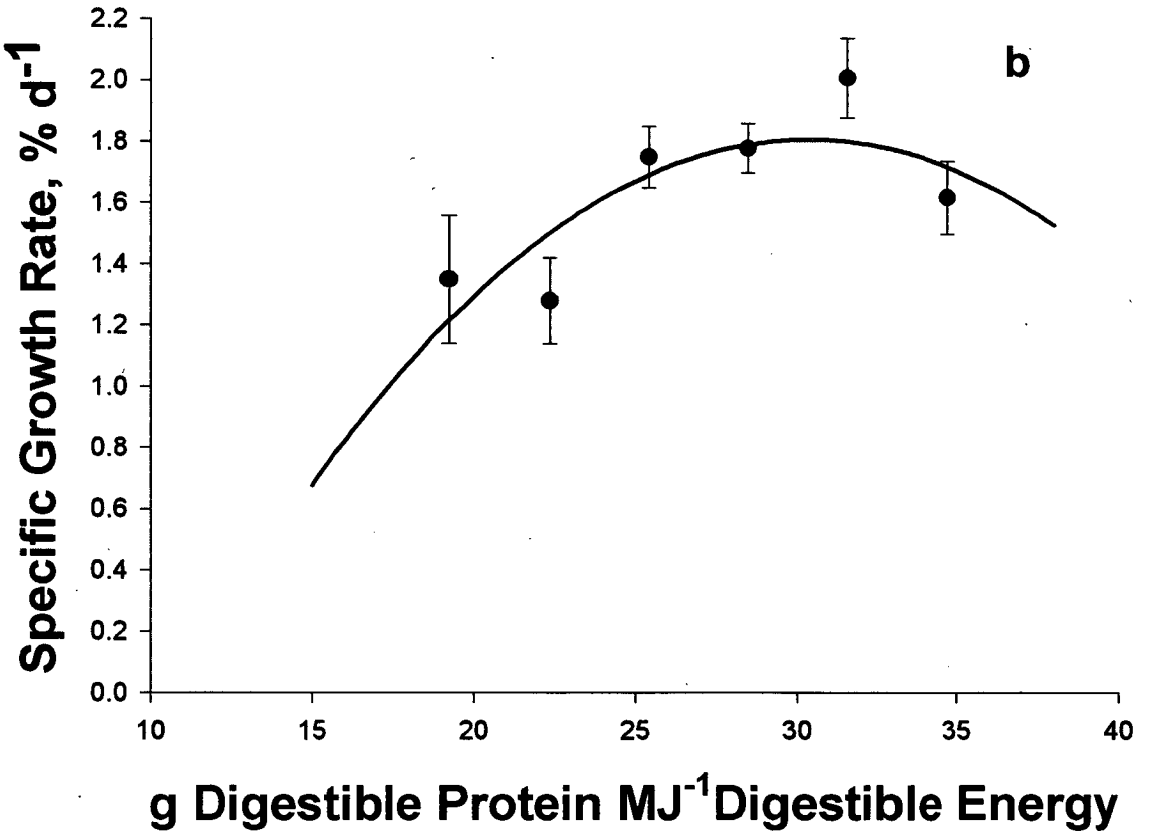
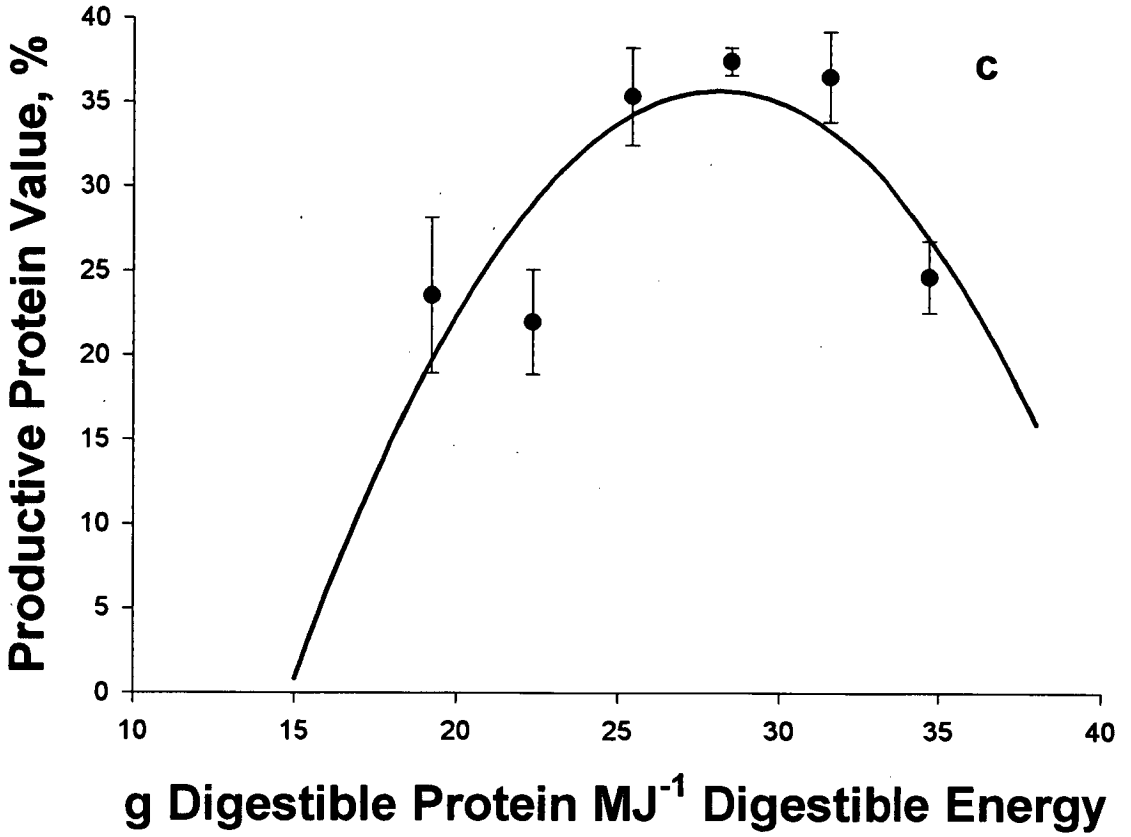
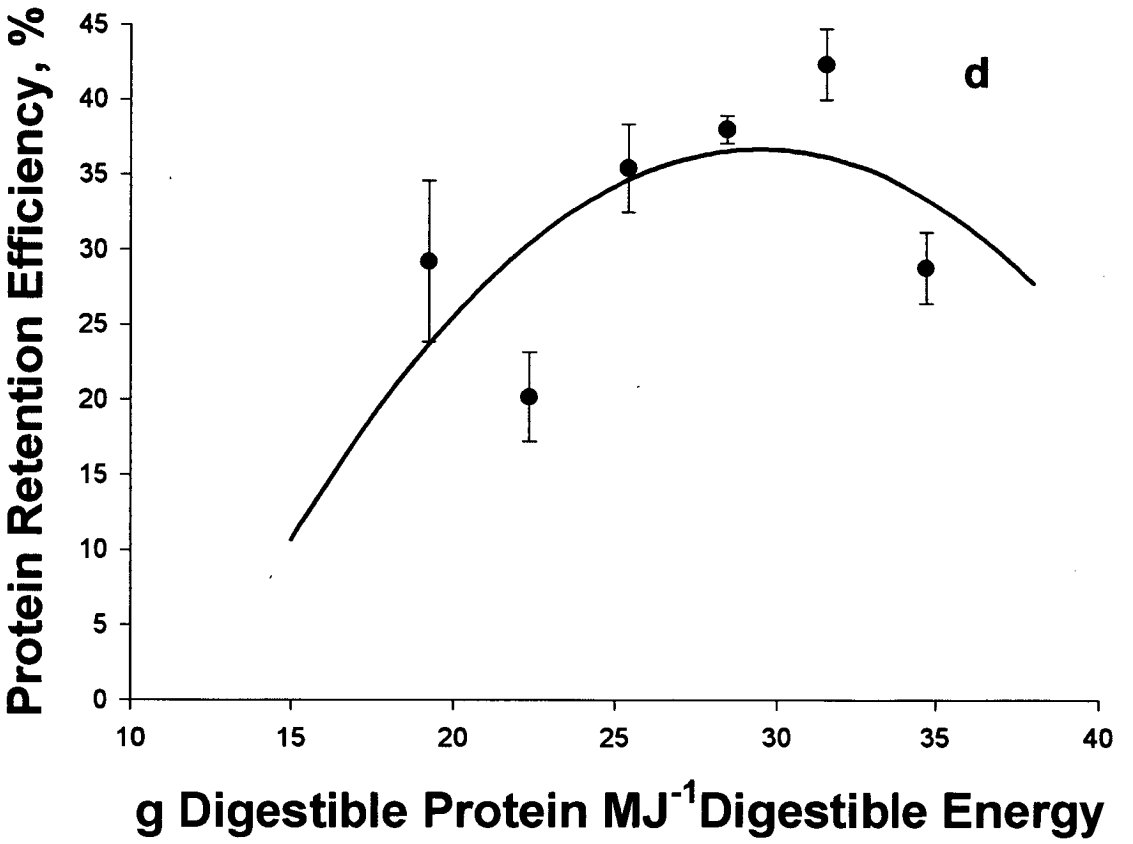

Protein Retention Efficiency, %
45
40
35
30
25
20
15
10
20
30
40
50
60
70
Dietary Protein Level, %
d

Figure 2.5. Fit of (a) % increase in body weight (b) specific growth rate (c) Productive protein value and (d) protein retention efficiency data to a second order polynomial (quadratic) model for estimation of protein requirement in terms of a digestible protein to digestible energy ratio. Model details are given in the text. Values are mean values ± S.E.


Body Weight Increase, %
300
280
260
240
220
200
180
160
140
120
100
a
10
15
20
25
30
35
40
g Digestible Protein MJ-1 Digestible Energy


Specific Growth Rate, % d-1
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
b
10
15
20
25
30
35
40
g Digestible Protein MJ-1Digestible Energy


Productive Protein Value, %
40
35
30
25
20
15
10
5
0
10
15
20
25
30
35
40
c
g Digestible Protein MJ-1 Digestible Energy


Protein Retention Efficiency, %
45
40
35
30
25
20
15
10
5
0
d
10
15
20
25
30
35
40
g Digestible Protein MJ-1Digestible Energy

CHAPTER 3
NITROGEN EXCRETION AND PROTEIN NITROGEN FLUX IN JUVENILE GREENBACK FLOUNDER

3.1 Introduction

Nitrogenous excretion rates have been recognized to be of importance in the study of nitrogen metabolism and balance in fish (Jobling 1981a). Nitrogen balance relates nitrogen consumed to nitrogen lost through waste products and nitrogen retained for growth (Birkett 1969). Ammonia is the major constituent of nitrogenous losses (Forster and Goldstein 1969, Randall and Wright 1987) although smaller quantities of other end products such as urea, trimethylamine oxide, creatine, creatinine, uric acid and amino acids are also excreted.

Nitrogen excreted consists of endogenous and exogenous fractions : the endogenous fraction is the basal level or lowest level of nitrogen excretion (Birkett 1969; Savitz 1969) and is due to the catabolism of body proteins. Exogenous excretion occurs as a result of feeding (Jobling 1981a) with nitrogenous products being formed following deamination of amino acids.

There have been numerous studies on the effect of feeding level (Savitz *et al.* 1977; Jobling 1981a; Kikuchi *et al.* 1991; Gershanovich and Pototskij 1992 Dosdat *et al.* 1995) and feed composition (Beamish and Thomas 1984; Carter and Brafield 1992; Forsberg and Summerfelt 1992; Li and Lovell 1992) on nitrogenous excretion There have been fewer studies on the effect of feeding

frequency on nitrogen balance and excretion rates (Ramnarine *et al.* 1987; Kaushik and Gomes 1988; Yager and Summerfelt 1994).

Intensive fish farming is accompanied by high levels of feeding of protein rich diets that result in the excretion of high levels of nitrogenous waste products. Construction of nitrogen budgets yield information on the effect of nutritional variables on nitrogen excretion rates and retention efficiencies. These are of value in formulating diets and in designing feeding regimes that promote growth and reduce pollution (Kaushik and Cowey 1991; Handy and Poxton 1993). Flatfish generally have a high dietary protein requirement, (Cowey *et al.* 1970; Bromley 1980; Caceres-Martinez *et al.* 1984; Guillaume *et al.* 1991), with relative concentrations of protein exceeding 45% of the total weight of the diet. The greenback flounder too has a high requirement for dietary protein, requiring a protein content of around 44-48% (refer to chapter 2 of this study).

In this study the excretion rates of both fasted and fed fish were estimated. Fed fish were subjected to different feeding levels and frequencies in order to study their effect on daily nitrogen excretion rates. Nitrogen excretion rates were used along with nitrogen consumption and faecal nitrogen loss to construct nitrogen budgets for fish maintained under different feeding regimes. During the excretion experiments, Total Nitrogen (TN) was measured in addition to Ammonia Nitrogen (TAN) and Urea Nitrogen (UN) that are usually measured. Increases in

feeding frequency have been reported to improve growth, conversion efficiency and water quality (Yager and Summerfelt 1994). There is an increase in food consumption accompanying increase in feeding frequency and it is of interest to investigate whether excretion and retention are influenced primarily by ration level. Therefore part of this study examined the effect of varying feeding frequency at a particular feeding level to examine if there was any effect on nitrogen retention efficiency.

The greenback flounder, *Rhombosolea tapirina* , is being considered as a potential species for culture in Australia (Hart 1994) . The objective of this study was to estimate excretion rates, construct nitrogen budgets and to model the fate of dietary nitrogen in fish fed at different levels and at different frequencies.

3.2 Materials and Methods

3.2.1 Experimental animals

Juvenile greenback flounder weighing 5-10 g were obtained from the Department of Primary Industry and Fisheries, Taroona, Tasmania and transported to the National Key Centre for Aquaculture, University of Tasmania, Launceston. The fish were held in circular 200 L Reln™ tanks that were supplied with recirculated seawater at 16°C. The light cycle was maintained at 12L:12D. The fish were fed on salmonid starter pellets (2mm; M/s Gibsons Feed Industries, Cambridge, Tasmania) twice a day to satiation. Water quality parameters such as dissolved oxygen, ammonia and salinity were routinely monitored and maintained at acceptable levels (Chin 1993; Forteath et al. 1993). The fish were maintained in the stock tanks for 21 days before being transferred to the experimental system.

3.2.2 Experimental system

The experimental system consisted of forty flat-bottomed 40-L rectangular plastic tanks that formed part of a recirculating seawater system. The tanks were white in colour and were equipped with lids to prevent fish from jumping into adjoining tanks. The tanks were arranged in two tiers and in two rows on a wooden frame. The tanks were surrounded by black PVC curtains in order to minimize

disturbance to the experimental fish. Each of the tanks was supplied seawater by means of an adjustable valve and drained by a L shaped outflow pipe into a common drain that carried water to a 2000 L Reln™ tank through a Dacron solids filter and a biofilter. Water from the sump was also pumped by means of a separate pump through a protein skimmer. Flow rates of water to each tank was maintained at approximately 4 L per minute. Each tank was supplied with an air line and an air stone thereby ensuring proper aeration. The temperature of the seawater was maintained at 16±0.5°C by controlling the temperature of the room. The photoperiod during experiments was set to a 12L:12D cycle by means of fluorescent lighting.

3.2.3 Excretion experiments

Thirty six fish were selected and distributed among experimental tanks with one fish being stocked per tank. The mean weight of fish used in the excretion experiments was 12.14± 1.14g. The fish in the experimental tanks were randomly assigned one of four experimental treatments. One group of six fish were fasted for measurement of basal or endogenous excretion rates. The other three groups of ten fish each were fed 1% body weight once per day, 0.5% body weight once per day and 0.5% body weight twice per day (Table 3.1). The fish were acclimated to specific experimental feeding regimes for a

period of two weeks during which time they were fed on a salmonid starter diet (2mm pellets; M/s Gibsons Feed Industries, Cambridge, Tasmania). The nitrogen content, moisture and ash content of the diet was analyzed as described earlier. The proximate composition of the diet is given in Table 3.2.

During experiments fish were fed at 0900 hrs and for a second time at 1300 hrs in the case of fish that were fed twice. Immediately after feeding the water flow to tanks involved in a particular experiment were turned off for a 24 hour period. The volume of water for experiments was set at 20L. At the start of the experiment a 200 ml sample of water was drawn out of the experimental tanks and acidified with a few drops of concentrated sulfuric acid. The containers were tightly capped and stored in a freezer at -20°C (Sayer and Davenport 1987). The airstones to each of the experimental chambers was left turned on to prevent oxygen deprivation. At the end of the 24 hour period another water sample was collected and treated in the manner described before. Following this the circulation to the experimental tanks was turned on again. This procedure was repeated for the three treatments for fed fish (n=10 for each treatment). In the case of fasted fish the fish were fasted for 48 hours before their excretion rates were analyzed (n=6). The water samples were analyzed for total nitrogen (TN), Total ammonia nitrogen (TAN) and Urea Nitrogen (UN).

In a separate experiment, two sets of tanks (n=2 each) were maintained over a 24 hour period without any fish in them. Water samples from the first set of tanks was analyzed for total ammonia nitrogen (TAN) at the start and at the end of the 24 hour period. The second set of tanks was spiked with approximately 5 ppm ammonium chloride. The water was analyzed for total ammonia nitrogen at the beginning and end of the 24 hour period. The ammonia concentrations were measured on a Hach spectrophotometer.

3.2.4 Analysis of water samples

Ammonia analysis

Ammonia in water samples was analyzed using the phenol hypochlorite method (Strickland and Parsons 1972; Stirling 1985). 15 ml portions of ammonia free seawater (3.5% solution of sodium chloride in distilled water) and ammonium chloride standards (made up in a 3.5 % sodium chloride solution) were treated with 0.6 ml of phenol solution (20 g in 200 ml of 95% v/v ethanol), 0.6 ml of sodium nitroprusside solution (1g in 200 ml of distilled water) and 1.5 ml of freshly prepared oxidizing reagent. The oxidizing reagent consisted of an alkaline reagent (100g sodium citrate and 5g sodium hydroxide in

500 ml of distilled water) and sodium hypochlorite (commercial bleach) mixed in a 4:1 ratio. Following the addition of the oxidizing reagent the samples were stored in a dark container for colour to develop. The samples were then read in a spectrophotometer at 640 nm to constuct a calibration curve for absorbance vs ammonia concentration. Water samples from experiments were subjected to a similar procedure and ammonia concentrations were estimated.

Urea analysis

Urea was estimated using a modification of the method used by Carter and Brafield (1991). The urea in samples was hydrolyzed using an urease solution and the excess ammonia measured was used to calculate the concentration of urea. An urease solution (100 IU ml^{-1}) was prepared by dissolving urease (Sigma U-4002) in a 0.5M sodium citrate buffer(adjusted to pH 7). 15 ml samples were incubated with 1.5 ml of urease solution in a water bath at 30°C for 30 minutes . The samples were brought down to room temperature and analyzed for ammonia by the phenol hypochlorite method. The ammonia evolved from the hydrolysis of urea was calculated with reference to a seawater blank (3.5% sodium chloride in distilled water) treated with urease and test water samples not treated with urease.

Total Nitrogen analysis

Total nitrogen was analyzed using the method of Kalff and Benzen (1984). This method employs potassium persulfate to oxidize all forms of nitrogen under pressure to form nitrate which is then treated with sodium salicylate in an acidic medium to produce nitrosalicylic acid. Nitrosalicylic acid produces a yellow colour with sodium potassium tartrate (Rochelle salt) under highly alkaline conditions and can be determined spectrophotometrically. 35 ml samples of water including blanks (3.5% sodium chloride solution), standard solutions of urea and test samples were measured into Schott™ bottles with screw top lids. 0.14 g of potassium persulfate powder was added to each bottle which was then loosely capped and autoclaved at 103 Kpa (15PSI) for 40 minutes. Following autoclaving, the bottles were cooled and 5 ml samples were taken from each bottle in Erlenmeyer flasks. To each flask 0.09 ml of 2M sulfuric acid was added followed by 2 ml of a 1% sodium salicylate solution. The flasks were covered with watch glasses and heated on a hot plate at 98°C with care being taken not to boil their contents. When dry 1 ml of concentrated sulfuric acid was added to each flask and swirled until the precipitate dissolved. The solution was left to stand for 10 minutes after which 20 ml of distilled water and 5 ml of highly alkaline Rochelle salt (sodium potassium tartarate) solution was added. The flasks were shaken and then left for

a yellow colour to develop which was read on a spectrophotometer at 420 nm. Total N concentrations were calculated with references to the seawater blank and standards.

3.2.5 Calculations

The daily rates of nitrogen excretion of fed and fasted fish was determined. Nitrogen consumed C_N, nitrogen excreted E_N(total Nitrogen) and nitrogen retained R_N, were calculated on a weight specific basis and are reported on a milligram per kilogram per day basis.

Faecal nitrogen F_N, was estimated using digestibility data from a study on the greenback flounder fed a similar salmonid diet (Chin 1993). The mean digestibility of nitrogen was calculated to be 95%.

The nitrogen retained was estimated using a nitrogen balance equation outlined in Birkett (1969):

$$C_N - F_N = A_N = R_N + E_N$$

where C_N= nitrogen consumed; F_N= nitrogen lost through faeces; A_N= nitrogen absorbed; R_N= nitrogen retained and E_N=nitrogen excreted.

The nitrogen retained was solved for by the difference between nitrogen consumed and nitrogen lost through faeces and excretion. The fraction of nitrogen consumed that was lost through excretion E_N/C_N and the fraction of nitrogen consumed that was retained, R_N/C_N were also calculated .

3.2.6 Statistical analysis

Nitrogen excretion rates and nitrogen retention from different feeding treatments were analyzed using a one way analysis of variance and treatment means were compared using the Tukey's test($p<0.05$). The proportions of consumed nitrogen that were excreted and retained were also analyzed by one way analysis of variance and their means compared . All the statistical analyses were carried out using SigmaStat ver. 3.0 (Jandel Scientific Co., San Rafael, California).

3.3 Results

3.3.1 Control tanks

The ammonia levels in unspiked tanks were below detectable limits both at the beginning and the end of the 24 hour period. In the tanks that were spiked with ammonium chloride, there was no significant difference (< 5%) between the total ammonia nitrogen concentrations at the beginning and end of the 24 hour period.

3.3.2 Excretion by fasted fish

The mean endogenous excretion rates of fasted juvenile greenback flounder are shown in Table 3.3. The mean daily total nitrogen (TN) excretion rate was found to be 23.36 ± 1.67 mgN kg^{-1} day^{-1}. The total ammonia nitrogen (TAN) excreted over a 24 hour period was estimated at 19.20 ± 1.21 mg N kg^{-1} day^{-1}. The ammonia nitrogen excreted constituted 82.5% 0f the total nitrogen excreted. Urea nitrogen (UN) was excreted at the rate of 2.49 ± 0.24 mg N kg^{-1} day^{-1}. Urea nitrogen formed approximately 10.4% of total nitrogen excreted . Ammonia and urea combined formed approximately 93% of the total nitrogen excreted.

3.3.3 Excretion by fed fish

There was a 9-18 fold increase in excretion rates of fed fish over fasted fish (Table 3.4). The mean total nitrogen (TN) excretion rates were 402.54 ± 7.66, 236.60 ± 3.99 and 398.34 ± 6.35 mgN kg^{-1} day^{-1} for fish from treatments A, B and C. The TN excretion rate of fish from treatment B was significantly lower than those from treatments A and C ($p<0.05$). There was no significant difference in the total nitrogen excretion rates of treatments A and C, although there was a minor decline in value for treatment C. Approximately 64% of ingested nitrogen was excreted by fish in treatment B (Table 3.4). The amount of nitrogen excreted by fish in treatment A and C were significantly lower and constituted approximately 54% of the nitrogen ingested.

The mean total ammonia nitrogen (TAN) excretion rate for fish from treatment B (197.24 ± 2.63 mgNkg^{-1} day^{-1}) was significantly lower ($p<0.05$) than those from treatment A (342.78 ± 7.26 mg Nkg^{-1} day^{-1}) and treatment C (334.71 ± 5.16 mg Nkg^{-1} day^{-1}). The total ammonia excretion rate of fish fed 0.5% body weight twice a day (treatment C) was lower (Table 3.4) than the rate in fish fed 1% body weight once a day (treatment A), but was not significantly different. Total ammonia nitrogen made up 83-85% of total nitrogen in all three treatments.

Urea was excreted at the rate of 36.88 ± 1.22 mg N kg^{-1} day^{-1} and 35.10 mgNkg^{-1} day^{-1} by fish from treatments A and C respectively. The urea excretion rate of treatment C fish was slightly lower than fish from treatment A but was not significantly different. The urea excretion rate of fish in treatment group B (25.67 ±1.71 mgN kg^{-1} day^{-1}) was significantly ($p<0.05$) lower than the other groups. Urea constituted 8-9% of total nitrogen excretion (Table 3.4). The rate of urea excretion in fed fish was 9-15 fold higher than in fasted fish. The urea excretion rate of fish was lower in fish that were fed a lower ration size (Table 3.4)

3.3.4 Nitrogen budgets for fed fish

The nitrogen budgets for fish maintained under the three experimental feeding treatments is given in Table 3.5. Nitrogen consumed in terms of mg N kg^{-1} day^{-1} was calculated from rations fed to fish. Faecal nitrogen F_N was calculated assuming a 95% nitrogen digestibility and therefore a 5% loss of nitrogen in the faeces. Nitrogen retention R_N was calculated using the nitrogen budget equation, $R_N = C_N - (F_N + E_N)$. E_N was determined experimentally as total nitrogen(TN) and R_N was solved by difference. The nitrogen retained by fish from treatments A (295.47 ± 8.13 mgNkg^{-1} day^{-1}) was lower than that seen in fish from treatment C (299.86±6.60 mgNkg^{-1} day^{-1}) but not significantly. The nitrogen retained by fish from group B was 111.76±3.89 mgNkg^{-1} day^{-1}

and was significantly lower than nitrogen retained in the other treatments.

Nitrogen retention efficiency was calculated in terms of nitrogen consumed R_N/C_N. 40.21% and 40.80% of consumed nitrogen was retained by fish from treatments A and C respectively. These efficiencies were not significantly different from one another. The retention efficiency of fish from treatment C was 30.52% and was significantly lower ($p<0.05$) than the retention efficiencies of treatment groups A and C (Table 3.5)

The flux of nitrogen is shown in Figure 3.1. The diagram shows the input of nitrogen, nitrogen losses and nitrogen retained. The diagram also shows the percentages of nitrogen lost and retained by fish from different treatments. Fish from treatment A lost 54.79% of ingested nitrogen by excretion and 5% through faeces while retaining 40.21%. Fish from treatment C showed a similar pattern of nitrogen loss(54.20%) and retention (40.8%). The percentage of retention in fish from group B was 30.52% with nitrogen excretion losses accounting for 64.48% of nitrogen consumed (Table 3.5).

3.4 Discussion

During the excretion experiments the volume of water in experimental tanks was kept at 20L. Such relatively large volumes of water for fish (mean weight approx. 12 g) was necessitated by the fact that the system was kept static so as to accumulate the excretions by fish over a 24 hour period. Lower volumes of water would have resulted in higher concentrations of ammonia during the experiments. The presence of a larger volume of adequately aerated water helped to decrease the stress on experimental fish. Measurements over shorter intervals may be error prone as excretion levels may not always be correctly estimated at low levels. The tanks were supplied with air through airlines and airstones and this may have been a potential source of nitrogen loss to the atmosphere. Brett and Zala (1975) estimated a loss of less than 1% through unionized ammonia. During experiments in the present study pH was around 7.8 and temperature was at 16°C and the percentage of unionized ammonia was less than 2% of total ammonia. Air was supplied at a very steady trickle in order to decrease the formation of spray which might have otherwise aided in the loss of ammonia. The loss of nitrogenous products by bacterial decomposition was minimized by the fact that the experimental tanks were static and not connected to a biofilter. In addition experimental tanks were scrubbed thoroughly thereby not allowing a substrate for

bacterial films to form. Another source of error is nitrogen contribution through the decomposition of feed and faeces. This contribution has been described to be small and not significant (Iwata 1970; Porter *et al.* 1987). During experiments uneaten food and faeces were siphoned out at regular intervals thereby minimizing the chance of nitrogen leaching into the water. The controls in this experiment showed that bacterial contribution to total nitrogen and bacterial degradation of nitrogen was negligible.

During this study, the ration size fed to each fish was kept constant and it was assumed that the nitrogen excretion rates during the 24 hour sampling window were representative of the daily nitrogen excretion rates. Day - to - day variation in excretion rates of fish fed a constant ration was not measured and hence this assumption was not tested.

While studying the nitrogen excretion rates of fish, most researchers have measured ammonia and urea excretion rates and totaled them to obtain a total nitrogen excretion rate. Few studies have determined total nitrogen in addition to ammonia and urea excretion rates (Durbin and Durbin 1981; Beamish and Thomas 1984; Kikuchi *et al.* 1991). The problem associated with measuring only ammonia excreted or ammonia and urea excreted is that it may not provide a complete picture of the nitrogen excreted. There are excretory products other

than ammonia and urea that may contribute to total nitrogen excretion (Forster and Goldstein 1969; Randall and Wright 1987). In the present study ammonia (TAN) and urea(UN) together made up 92-94% of the total nitrogen excreted. This would mean that approximately 6-8% of total N was contributed by other forms of excretory nitrogen. While this is relatively small it can contribute to errors in the modelling of protein nitrogen flux.

Ammonia formed 82.5% of the total nitrogen excreted by juvenile greenback flounder in this study. Urea excreted was approximately 10% of the total nitrogen excreted. This is in agreement with excretion patterns seen in other freshwater and marine fish (Forster and Goldstein 1969; Brett and Zala 1975; Elliott 1976; Jobling 1981; Randall and Wright 1987; Handy and Poxton 1993). Endogenous rates of fish have been estimated using different approaches. Some workers have measure it on fish after short periods of fasting (Iwata 1970; Savitz *et al.* 1977; Cui and Wootton 1988; Carter and Brafield 1992) while others have measured it after feeding fish on protein free diets (Gerking 1955 b.;Savitz 1969). A third approach has used the relationship between nitrogen intake and nitrogen retention (Gerking 1955a; Jobling 1981). Comparisons of endogenous excretion rates between species are complicated by this and by the use of different fish sizes, experimental temperatures and periods of starvation. have shown An initial decrease in nitrogen excretion rate is followed by a

stabilization around six days after the onset of starvation (Brett and Zala 1975; Dosdat *et al.* 1995). Dosdat *et al.* (1995) observed a stabilization in TAN rates two days after the onset of fasting.

Jobling (1981) reported an endogenous excretion rate of 27 mg kg^{-1} day^{-1} for 5-90 g plaice and the results from this study are close in value to it. Birkett (1969) found rates of 55.92 and 207.84 mg kg^{-1} day^{-1} for 16-28 g and 0.7-3.7 g plaice respectively. Sole weighing 6-53 g and 0.6-1.8g had rates of 148.8 and 120 mg kg^{-1} day^{-1} respectively (Birkett 1969). The rate in the dab was 176.64 mg kg^{-1} day^{-1} (Sayer and Davenport 1987) . In turbot weighing 13.5 g the endogenous rate of ammonia nitrogen and urea nitrogen was found to be 49.3 and 9.6 mg N kg^{-1} day^{-1} (Dosdat *et al.* 1995). In the case of starved Japanese flounder (Kikuchi *et al.* 1990) the rates of ammonia and urea excretion were 54 and 17.4 mg kg^{-1} day^{-1} respectively. Endogenous excretion rates of various fish species have been reviewed (Brett and Zala 1975; Gershanovich and Pototskij 1992; Dosdat *et al.* 1995) and the endogenous rates of excretion of the greenback flounder appear at the lower end of the range of published values.

Nitrogen excretion rates showed an increase after feeding and is as seen in other fish (Brett and Zala 1975; Elliott 1976 ; Rychly and Marina 1977; Lied and Braten 1984). There was a 9-18 fold increase in nitrogen excretion rates of fed flounder over those of fasted flounder.

The observed magnitude of increase is higher than that seen in sea bream (Echevarria *et al.* 1993), Asian sea bass (Alamendras 1994), Japanese flounder (Kikuchi *et al.* 1991) and plaice (Jobling 1981).

Rates of total nitrogen, ammonia and urea excretion were lower in fish fed the lower ration size of 0.5% body weight per day (treatment B) than fish fed 1% body weight per day (treatments A and C). The rates of excretion of treatments A and C were similar although the treatment where 1% was fed in two 0.5% portions had a slightly lower excretion rate. The excretion rates in fish fed the 0.5% body weight ration were lower because of the lower amount of nitrogen ingested. It is therefore perhaps more objective to compare the percentages of consumed nitrogen that have been excreted and retained to gain a better understanding of the flow of nitrogen through the fish.

The fraction of consumed nitrogen excreted (E_N/C_N) was higher in the treatment where fish were fed a lower ration. The E_N/C_N value for fish from treatment B was significantly lower than that for fish from treatments A and C. This explains the excretion rates in terms of nitrogen consumed and may facilitate comparisons between fish maintained under similar conditions.

Even though it is difficult to compare results from different studies it is possible to compare general trends. In general increase in ration size

has been accompanied by an increase in nitrogen excretion. Jobling (1981) observed an increase in excretion rates with quantity of digestible nitrogen fed. In the brown trout *Salmo trutta*, there was an increase in the energy lost in excretory products with increasing ration size(Elliott 1976). Beamish and Thomas (1984) found that total nitrogen excretion increased in proportion to ingested nitrogen. They also found that the proportion of ingested nitrogen that was excreted decreased with increasing ration size.

Similar increases in excretion rates with increase in ration size have been found in several other studies (Dosdat *et al.* 1995; Carter and Brafield 1992; Kikuchi *et al.* 1992; Savitz *et al.* 1977; Gershanovich and Pototskij 1992). Gershanovich and Pototskij (1992) observed an increase in excretion rates with an increase in ration size from 2.2% body weight per day to 8.4% body weight per day before it declined at higher feeding levels.

There are contrasting lines of evidence on the effect of ration size on urea excretion in fish. Ration size was not found to increase urea excretion in salmon (Brett and Zala 1975), rainbow trout (Beamish and Thomas 1984) , and sturgeon (Gershanovich and Pototskij 1992). It was found to increase urea excretion rates in flatfish species such as turbot (Dosdat *et al.* 1995) and the Japanese flounder (Kikuchi *et al.* 1992). In the present study urea excretion rates increased with an

increase in ration size (Table 3.4). The urea excretion rates in fish fed 0.5% body weight were lower than those fed 1% body weight per day. When viewed as a proportion of total nitrogen excreted, there was no difference in the urea excretion rate at different feeding levels with urea nitrogen contributing roughly 9% of total nitrogen excreted.

There was an increase in nitrogen excretion rates with an increase in the number of feeds per day but this was not significantly greater (Table 3.4). The proportion of consumed nitrogen excreted was not significantly different between groups of fish fed once or twice daily (Table 3.5). This was probably due to the fact that fish from both groups (treatments A and C) were fed the same ration size (1% body weight per day); ration size is more important than the number of times the fish are fed. Yager and Summerfelt (1994) fed walleye fingerlings to satiation 1, 2, 3 and 15 times per day. The mean ammonia excretion rates at 3 feedings per day was the only one that was significantly higher than the other levels. In cod (Ramnarine *et al.* 1987) fish fed more than once a day showed a cumulative effect on excretion rates. Kaushik and Gomes(1988) fed rainbow trout 0.5% d^{-1}, 1% every 2 days and 2% every 4 days. The total nitrogen excretion was highest in the treatment where fish were fed 2% body weight every 4days. The ratio of nitrogen excreted in relation to nitrogen consumed was similar in all three treatments.

The nitrogen budgets constructed for fish under the different feeding treatments (Table 3.5) showed that there was a significant difference in excretion rate and retention efficiency of nitrogen consumed due to increase in ration size. An increase in ration size , while causing an increase in weight specific excretion rate , resulted in a significantly lower proportion of consumed nitrogen being excreted and a significantly higher retention of consumed nitrogen. The increasing of number of feeds during the day did not produce a significant change in the retention efficiency of nitrogen. The value of the retention efficiency of consumed nitrogen nitrogen at the 0.5% ration size feeding level were 30% while the efficiency at 1% ration level was 40% (Figure 3.1). Gerking (1971) found a linear increase in retention efficiency with increase in nitrogen consumption at lower levels of feeding in bluegill sunfish. Gerking (1955) however expressed the view that at higher feeding levels retention efficiency would approach a constant value. Savitz *et al.* (1977) found an increase in retention efficiency with increase in nitrogen consumption followed by a levelling off at higher ration levels in the smallmouth bass, *M. salmoides*. This study studied retention efficiency at only two ration levels and therefore the eventual levelling off of retention efficiency at higher ration levels was not observed. Retention efficiency did not differ significantly at different frequencies at the same feeding level showing that retention was not affected when the ration level was kept constant. Kaushik and Gomes (1988) observed a significant increase in retention

efficiency in trout fed maintenance rations once every two days compared to trout fed similar rations once a day and every four days.

There is a great deal of variation in reported values of nitrogen retention efficiencies of different species in the literature. Jobling (1981) estimated a retention efficiency (of digestible N) of 18% from the relationship between nitrogen retention and digestible nitrogen. The values for white steenbras, *Lithognathus lithognathus* were higher at 85.3 and 90.7% consumed and absorbed nitrogen for small fish and 84.8 and 89.9% for large fish (Harris and Probyn 1996). Values in planktivorous fish were 41.5 and 47.8% in the cape anchovy (James *et al.* 1989) and 38.5% and 34.5% in the Atlantic menhaden (Durbin and Durbin 1981). Retention efficiencies for plaice (Birkett 1969) were 27-41%, for sole 39.1-49% (Birkett 1969) and 34-49% in Atlantic salmon (Carter *et al.* 1992).

Nitrogen retention, R_N in the present study was estimated by the difference between nitrogen consumed, C_N and nitrogen lost through faeces F_N and excretion, E_N. The accuracy of R_N is dependent on the accuracy of the estimation of C_N, F_N and E_N. C_N was determined by visual observation and a possible source of error is the loss of fine particles in the ingestion process. E_N was also determined experimentally and the sources of error have been discussed previously. Faecal nitrogen loss, F_N was calculated using digestibility

data from earlier work on greenback flounder. Despite reservations expressed in the literature (Jobling 1983) over the validity of extending digestibility data from one study to another, the use of Chin's (1993) data is valid for several reasons. The species of fish, size of fish, experimental temperature and diet composition in both studies were very similar thereby making the use of digestibilty data valid. It is therefore, logical that errors in the components of the nitrogen balance equation will have an effect on estimates of nitrogen retention. It is similar to nitrogen retention estimates from carcass analysis which is also dependent on accuracy of analysis.

In conclusion , ration size demonstrated a significant effect on the rate of nitrogen excretion and efficiency of nitrogen retention in greenback flounder. There was an increase in weight specific nitrogen excretion with increase in feeding level. The proportion of consumed nitrogen excreted decreased with increase in ration size as did the efficiency of retention of consumed and absorbed nitrogen. Increase in feeding frequency at the same feeding level did not produce any significant change in excretion rates or retention efficiency. The effects of a wider range of ration sizes and feeding frequencies on protein nitrogen flux needs to be examined. Nitrogen budgets and nitrogen flux models can be used as a tool in the understanding of the efficiency of utilization of various feeds and in the planning of feeding regimes at the optimum dietary protein level.

TABLE 3.1. Summary of experimental treatments

TREATMENT	No. of fish	FEEDING REGIME
A	10	1% Body weight once a day
B	10	0.5% Body weight once a day
C	10	0.5% Body weight twice a day
E	6	Fasted fish

TABLE 3.2. Composition of diet fed to juvenile greenback flounder

Component	% dry weight
Nitrogen	7.35[1]
Crude Protein	45.94[2]
Crude Fat	15.4[3]
Crude Fibre	1.85[3]
Ash	10.26[4]
Moisture	4.69[5]

[1] kjeldahl Nitrogen
[2] nitrogen*6.25
[3] information supplied by manufacturer
[4] ashed in a muffle furnace at 550°C for 16 h
[5] oven dried at 70°C to constant weight

TABLE 3.3. Endogenous excretion rates of juvenile greenback flounder. Fish were fasted for two days . Values reported are means ± SE for 6 fish.

Body weight , g	12.32 ± 0.38
Total Nitrogen (TN), mg kg^{-1} day^{-1}	23.36 ± 1.67
Total Ammonia Nitrogen (TAN), mgkg^{-1} day^{-1}	19.20 ± 1.21
Urea Nitrogen (UN), mgkg^{-1} day^{-1}	2.49 ± 0.24
TAN:TN	0.8250 ± 0.009
UN:TN	0.1038 ± 0.004

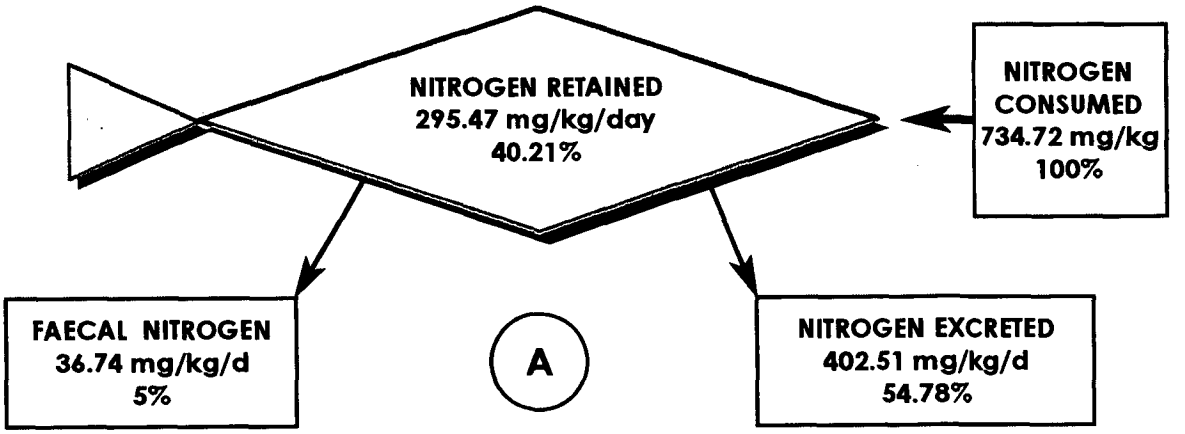
TABLE 3.4. Exogenous nitrogen excretion rates of fed juvenile greenback flounder. Values are means±SE for ten fish. TN = Total Nitrogen, TAN = Total Ammonia Nitrogen, UN = Urea Nitrogen. Values within a row with different superscripts are significantly different($p<0.05$).

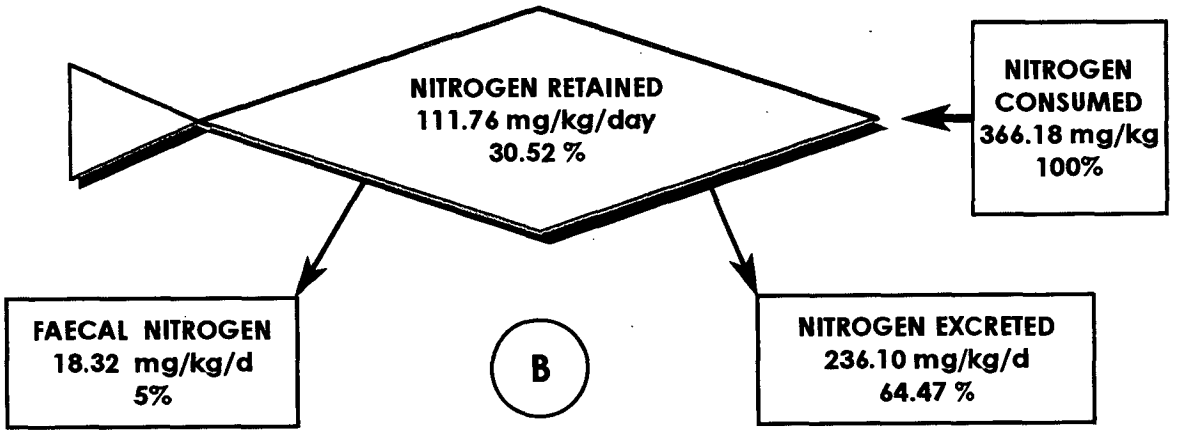
	TREATMENT A	TREATMENT B	TREATMENT C
Fish Weight, g	11.54 ± 0.23	12.22 ± 0.33	12.5 ± 0.25
TN, mg N kg^{-1} d^{-1}	402.54 ± 7.66^{a}	236.60 ± 3.99^{b}	398.34 ± 6.35^{a}
TAN, mg N kg^{-1} d^{-1}	342.78 ± 7.26^{a}	197.24 ± 2.63^{b}	334.71 ± 5.16^{a}
UN, mg N kg^{-1} d^{-1}	36.88 ± 1.22^{a}	23.18 ± 1.71^{b}	35.10 ± 0.79^{a}
TAN:TN	0.85 ± 0.005	0.83 ± 0.008	0.84 ± 0.003
UN:TN	0.091 ± 0.002	0.097 ± 0.005	0.088 ± 0.002

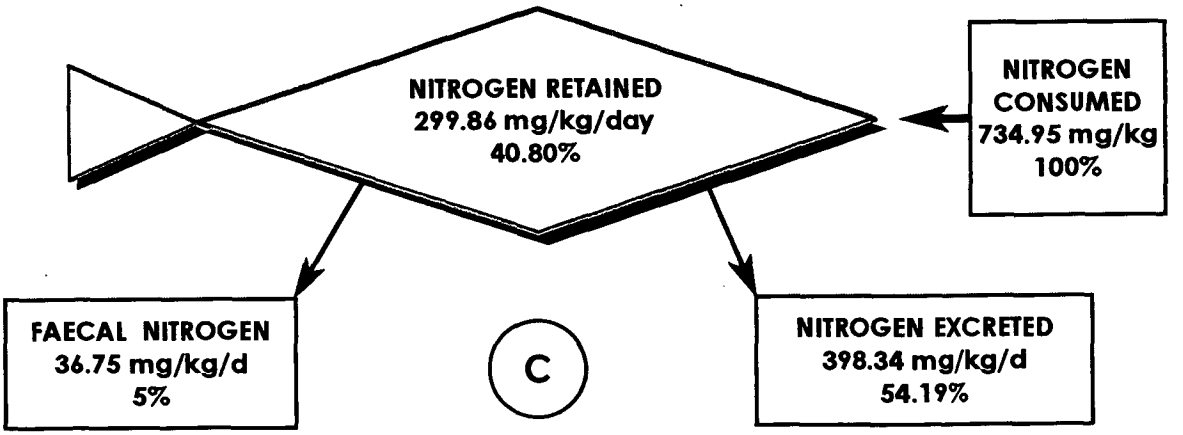
TABLE 3.5. Nitrogen budgets for fed juvenile greenback flounder maintained under different feeding regimes. Values are means ± SE of ten fish. C_N = Nitrogen consumed, F_N = Faecal Nitrogen, E_N = Nitrogen excreted, R_N = Nitrogen retained, R_N/C_N = Gross nitrogen retention efficiency, E_N/C_N = proportion of consumed nitrogen excreted. Values within a row with different superscripts are significantly different ($p<0.05$)

	TREATMENT A	TREATMENT B	TREATMENT C
C_N, mg kg^{-1} d^{-1}	734.72 ± 0.83	366.18 ± 0.71	734.95 ± 1.22
F_N, mg kg^{-1} d^{-1}	36.73 ± 0.04	18.30 ± 0.03	36.74 ± 0.06
E_N, mg kg^{-1} d^{-1}	402.51 ± 7.66^a	236.10 ± 3.99^b	398.34 ± 6.35^a
R_N, mg kg^{-1} d^{-1}	295.47 ± 8.13^a	111.76 ± 3.89^b	299.86 ± 6.60^a
R_N/C_N	0.4021 ± 0.01^a	0.3052 ± 0.01^b	0.4080 ± 0.009^a
E_N/C_N	0.5479 ± 0.01^a	0.6448±0.01^b	0.5420 ± 0.009^a

FIGURE 3.1. Protein -Nitrogen flux diagrams showing the fate of consumed nitrogen in juvenile greenback flounder fed (A) 1% body weight once a day (B) 0.5% body weight once a day (C) 0.5% body weight twice a day.. Values are treatment means ± SE for ten fish. Percentage values are in relation to nitrogen consumed.


NITROGEN RETAINED
295.47 mg/kg/day
40.21%
NITROGEN CONSUMED
734.72 mg/kg
100%
FAECAL NITROGEN
36.74 mg/kg/d
5%
A
NITROGEN EXCRETED
402.51 mg/kg/d
54.78%


NITROGEN RETAINED
111.76 mg/kg/day
30.52 %
NITROGEN CONSUMED
366.18 mg/kg
100%
FAECAL NITROGEN
18.32 mg/kg/d
5%
B
NITROGEN EXCRETED
236.10 mg/kg/d
64.47 %


NITROGEN RETAINED
299.86 mg/kg/day
40.80%
NITROGEN CONSUMED
734.95 mg/kg
100%
FAECAL NITROGEN
36.75 mg/kg/d
5%
C
NITROGEN EXCRETED
398.34 mg/kg/d
54.19%

CHAPTER 4
GENERAL DISCUSSION

4. General Discussion

It is recognized that fish do not have a requirement for protein *per se*, but rather for a well balanced mixture of amino acids (Wilson and Halver 1986). Energy intake is deemed a basic nutritional requirement because of its role in the maintenance of life processes which is given precedence over growth (NRC 1993). Despite these facts, the requirement for protein is typically given first consideration in the formulation of diets because of its expensive nature. An ideal balance of both protein and energy is required to promote optimal growth. Diets that are deficient in energy relative to protein will result in lower growth efficiency as protein will be metabolized to provide for maintenance. If the diet contains excess energy relative to protein it can inhibit protein intake and/or result in fat deposition. Fish require a higher protein to energy ratio than do terrestrial animals and it is thought that it does not necessarily indicate a higher protein requirement but rather a lower energy requirement for maintenance. The energy requirement in fish is lower because they do not require energy for temperature regulation and for the excretion of synthesized waste products (Tacon and Cowey 1985; NRC 1993).

It is necessary to define protein requirement and to distinguish between its various interpretations. Protein requirement has generally been

defined as the minimum amount of protein required to meet amino acid requirements and to achieve maximum growth (NRC 1993, Tacon and Cowey 1985; Jobling 1994). The approach used has been to feed fish diets containing graded levels of protein and to estimate requirement through the use of analysis of variance techniques or by constructing dose response curves and fitting them to regression models. Typically data on weight gain and growth rate have been used, although other measures such as protein utilization efficiency can be used just as effectively. Protein requirement is normally expressed as the percentage of the dry diet, although the use of digestible protein to digestible energy ratios is also prevalent (Garling and Wilson 1976; Daniels and Robinson 1986; Gurure *et al.* 1995). Jobling and Wandsvik (1983) expressed protein requirement in terms of a protein energy to total (gross) energy ratio.

It has been suggested that percentage composition and protein energy ratios may not be adequate in comparing protein requirement of different species and studies from different laboratories (Tacon and Cowey 1985). The authors expressed the view that requirements expressed solely as dietary percentages or protein energy ratios were of limited value and would be of greater use if they were related to food intake and growth. Daily protein requirement expressed in terms of protein consumed per

unit weight of fish per day was recommended as an alternative. The protein requirement is the protein consumed daily at the highest growth level. Tacon and Cowey (1985) found a strong linear relationship between daily protein requirement and specific growth rate for data from a variety of species. The linear relationship implies that protein utilization efficiency is relatively constant despite the fact that the data was from fish of different trophic modes. The relationship also suggested that the protein requirement of fish is not very different from terrestrial animals. The equation y = -1.342+ 7.586x described the relationship between specific growth rate and daily protein requirement for the data compiled by Tacon and Cowey (1985).The intercept suggests a maintenance daily protein requirement for 1.342 g kg^{-1} day^{-1} while the slope suggests that an additional 7.586 g kg^{-1} day^{-1} would be required for an additional 1% increase in specific growth rate

This generalized relationship predicts a daily protein requirement of 13.84 g kg^{-1} day^{-1} for greenback flounder at the maximum specific growth rate of 2.01 % day^{-1}. However, the observed mean daily protein intake at the maximum specific growth rate of 2.01% day^{-1} as estimated over the duration of the trial was 5.84g kg^{-1} day^{-1}. In comparison daily requirements for other flatfish species such as the plaice, *Pleuronectes platessa*, was 7.50 g kg^{-1} day^{-1} at the maximum specific growth rate of

0.93% d^{-1} and 19 g kg^{-1} day^{-1} in the turbot, *Scopthalmus maximus* at a maximum specific growth rate of 3.78 (Bromley 1980).

The disadvantage of using daily requirement is that it expresses requirement in absolute terms. This introduces a great deal of variability when fish of different physiological ages and from different environmental conditions are compared. Fish under different conditions and at different physiological ages differ in their growth potentials, use protein differently and are affected by environmental variables to varying degrees (Bowen 1987).

Fish, like other animals, have a maintenance requirement for protein. This is described as the protein intake required to maintain nitrogen equilibrium (Wilson 1989). At nitrogen equilibrium the nitrogen intake balances nitrogen losses and there is no net difference in body weight. Proteins in animal cells are constantly being recycled and some of the amino acids are removed through oxidation. This constitutes the absolute minimum requirement for protein that has been lost. The endogenous nitrogen losses of fish have been estimated by measuring total nitrogen excretions or through comparative carcass analysis. In the case of measurement of excretions fish are fasted, fed on low nitrogen or nitrogen free diets. In the case of carcass analysis, nitrogen retention is

measured by comparing the composition of fish before and after the experimental period. Endogenous nitrogen losses and maintenance requirements can also be estimated from the relationship between nitrogen intake and nitrogen retention (Boorman 1980; Jobling 1981a).

The endogenous nitrogen excretion rate for juvenile greenback flounder weighing approximately 12 g was experimentally determined to be 23.36 mg kg^{-1} day^{-1} (refer to chapter 3). This value multiplied by 6.25 yields the approximate maintenance protein requirement value of 0.15 g kg^{-1} d^{-1}. This is the minimum quantity of protein required to replace protein lost due to the basal metabolism of the fish in order to keep it in nitrogen balance or equilibrium. Comparative carcass analysis data from the protein requirement study (chapter 2) were used to plot the relationship between nitrogen consumed and nitrogen retained for flounder weighing approximately 1 g which was the mean weight of fish at the halfway mark of the trial. In the relationship between nitrogen intake and nitrogen retention (Boorman 1980) the y intercept is the endogenous (faecal and excretory) nitrogen loss. This quantity represents the loss of nitrogen through excretions and metabolic faecal loss which occurs even when fish are not fed (zero intake). The x intercept is an estimate of maintenance nitrogen requirements or the weight specific intake rate of nitrogen required to maintain nitrogen equilibrium (at zero growth). A

linear model of the form y=a+bx was fitted to the data and the relationship was described by the equation y = -333.84+0.624x. The endogenous nitrogen excretion rate of 1g fish as determined by this method was 333.84 mg N kg^{-1} d^{-1}. The endogenous loss in terms of protein can be calculated to be 2.06 g kg^{-1} d^{-1}. The maintenance nitrogen requirement was estimated at 533 mg N kg^{-1} d^{-1}. In terms of protein requirement the maintenance requirement at zero growth for 1g fish was estimated to be 3.31 g kg^{-1} d^{-1}. The maintenance requirement as estimated from endogenous losses at zero intake was lower than the maintenance requirement at zero growth. Jobling (1981a) plotted the relationship between absorbed nitrogen and retained nitrogen in juvenile plaice and found that endogenous excretion rate (0.27 g kg^{-1} day^{-1}) was lower than the maintenance nitrogen requirement of 0.15 g kg^{-1} day^{-1}. Jobling (1981a) compared these results with results from growth trials with plaice (Birkett 1969; Cowey *et al.* 1972) and found them to be similar after correcting for differences in fish size and temperature. Jobling (1981a) found endogenous excretion rates in post- absorptive fish to be intermediate in value to endogenous requirements and maintenance requirements and suggested that excretion rates of post absorptive fish or fasted fish did not provide accurate estimates of either basal or maintenance requirements.

The concept of maintenance requirement has a practical application in aquaculture. Maintenance requirement data is useful in the formulation of feeds for fish in the pre-harvest stage. Fish at this stage have attained their market size and any further growth is not required and food supplied in excess of maintenance needs would be economically wasteful. Another potential application is in the planning of feeding strategies that do not result in weight losses while reducing labour costs involved in feeding.

Protein-nitrogen flux is another approach that can be used along with weight gain and growth rate data to estimate protein requirement. Protein-nitrogen flux is the flow of ingested nitrogen through the body and relates nitrogen ingested with nitrogen retained and nitrogen lost .The data required for the construction of a protein-nitrogen flux is in essence the same as that required for a nitrogen balance model (Birkett 1969) or a nitrogen budget (Carter and Brafield 1992). The basic form of a protein nitrogen flux model can be written as:

N consumed = N retained + N lost (N lost = Faecal N + Excretory N)

Nitrogen retained is either directly determined through comparative carcass analysis or by difference after the other components have been experimentally determined

Protein-nitrogen flux can be used to corroborate both absolute and relative estimates of protein requirement. It takes into account nitrogen intake (food intake), nitrogen retention (growth) and nitrogen excretion. It therefore provides information on the utilization of dietary protein-nitrogen at various dietary protein concentrations and feeding regimes. It also offers an opportunity to jointly monitor nitrogen retention and nitrogen excretion . This is of great utility in the formulation of diets that promote growth while being environmentally friendly.

Protein-nitrogen flux models were constructed for flounder weighing approximately 12 g. These flounder were maintained under different feeding conditions (chapter 3). The flux diagrams (Figure3.1) show nitrogen retention and nitrogen loss both in absolute terms (mg kg^{-1} day^{-1}) and in relative terms as a proportion of nitrogen ingested. The nitrogen retained by fish fed on a salmonid diet containing 46% protein and fed 1% B.W. a day was roughly 40%. The proportion of dietary nitrogen retained was 30% when fed 0.5% B.W. a day. There was no significant difference in retention between groups of fish fed the same ration level at different frequencies showing excretion and retention to be more sensitive to ration size.

Flux diagrams were used for juvenile greenback flounder weighing 1g (Figure 4.2) to compare protein utilization and nitrogen excretion at various dietary protein levels. Nitrogen retention efficiency at 40, 45 and 50% dietary protein levels was generally higher than that seen at the 30,35 and 55% levels. Excretion rates at 30, 35 and 55% levels suggest that protein was being used for energy rather than growth. At higher dietary protein concentrations, the proportion of ingested nitrogen excreted was lower while the proportion that was retained was higher. Optimal retention and low nitrogen excretion rates were observed at the 45 and 50% protein diets.

It is difficult to compare the endogenous excretion rates arrived at using the two approaches, i.e. from direct monitoring of excretions of fasted fish and through extrapolation of the nitrogen intake - nitrogen retention relationship, due to the lack of a scaling relationship. It is however possible to compare the retention efficiencies of fish that were fed using the two approaches. The nitrogen retention efficiency of fish fed a 46% protein diet (Fig. 3.1), as determined by the difference between nitrogen consumed and nitrogen losses, was approximately 40%. The retention efficiencies for fish fed 40-50% protein diets (Fig. 4.2), as estimated by carcass analysis, were 35-37%. The results from the two approaches employed are in general agreement.

The results from this study suggest that greenback flounder require a high proportion of protein in their diets and also require a relatively high digestible protein to digestible energy ratio. This may not however mean that the greenback flounder and other flatfish that display similar requirements have a high requirement for protein. Bowen (1987) compared the protein requirement for maintenance, relative protein concentration for maximum growth, protein intake rate for maximum growth, protein retention efficiency and protein efficiency ratio between fish and terrestrial vertebrates. He found that fish differed only in their requirement for a higher relative protein concentration in their diets for maximum growth. Bowen (1987) further suggested that this high requirement was due to the relatively lower energy requirement of fish and that it did not by itself constitute a higher requirement for protein in fish. A possible reason for the requirement for higher dietary protein concentrations in flatfish is that they are generally sedentary and therefore have a lower requirement for energy. This lower energy requirement is expressed as a higher dietary protein concentration and protein to energy requirement.

In conclusion, the dietary protein concentration that promoted optimal growth was estimated to lie between 44 and 48%. The digestible protein to digestible energy ratio for maximum growth was estimated to be 29-30

g DP/MJ DE. Estimates of protein requirement from weight gain, specific growth rate and protein utilization were found to be in general agreement. The daily weight specific protein requirement for maximum specific growth rate for 1g was calculated to be 5.84 g kg^{-1} day^{-1}. The maintenance requirement of similar sized fish was estimated at 3.31 g kg^{-1} day^{-1}. Optimal protein utilization was observed when fish were fed diets containing 45-50% protein and protein nitrogen flux models showed that for diets containing 45-50% protein, nitrogen excretion as a proportion of ingested nitrogen was lowest while nitrogen retention was at its maximum.

The maintenance protein requirement of flounder weighing 12g as determined from the endogenous nitrogen excretion rate was estimated to be 0.15 g kg^{-1} day^{-1}. Nitrogen retention was affected more by ration size than by feeding frequency. Fish fed 1% body weight per day retained 40% of nitrogen consumed whereas fish fed 0.5% body weight per day retained 30% of consumed nitrogen. There was no significant difference in nitrogen retention efficiency between fish fed once or twice a day at a ration level of 1% body weight per day.

Figure 4.1. The relationship between nitrogen retained and nitrogen consumed for juvenile flounder weighing approximately 1 g. The equation of the linear fit is y= -333.08+0.624x. The y intercept (333.08 mgN kg^{-1} d^{-1}) is the value for endogenous nitrogen losses (excretory and faecal). The x intercept is the maintenance requirement (533 mg N kg^{-1} d^{-1}).

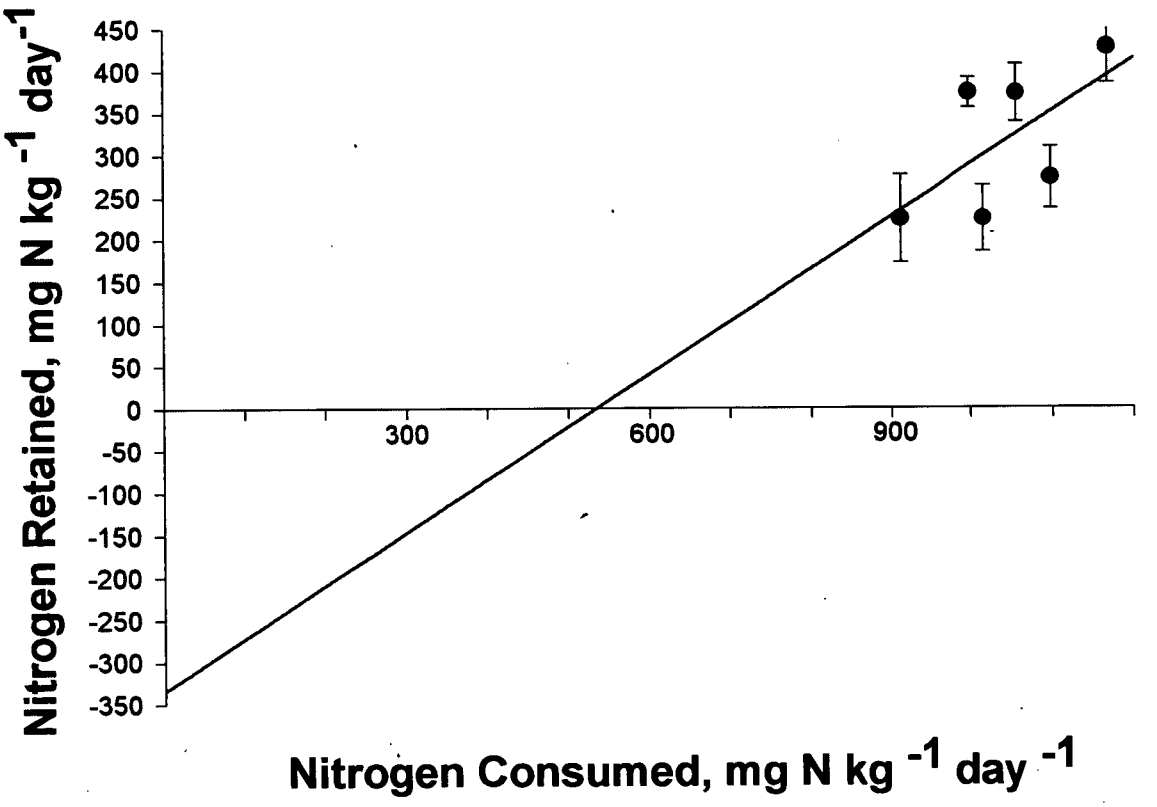
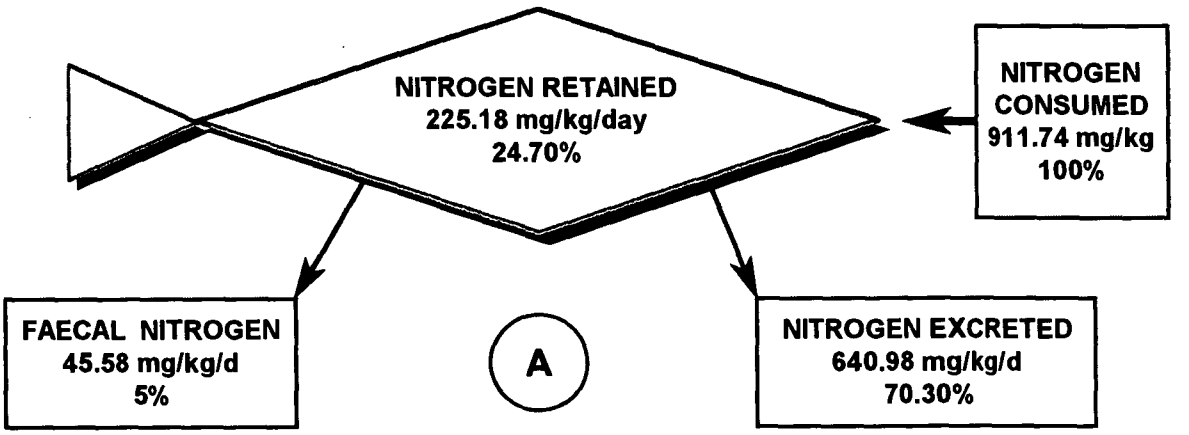
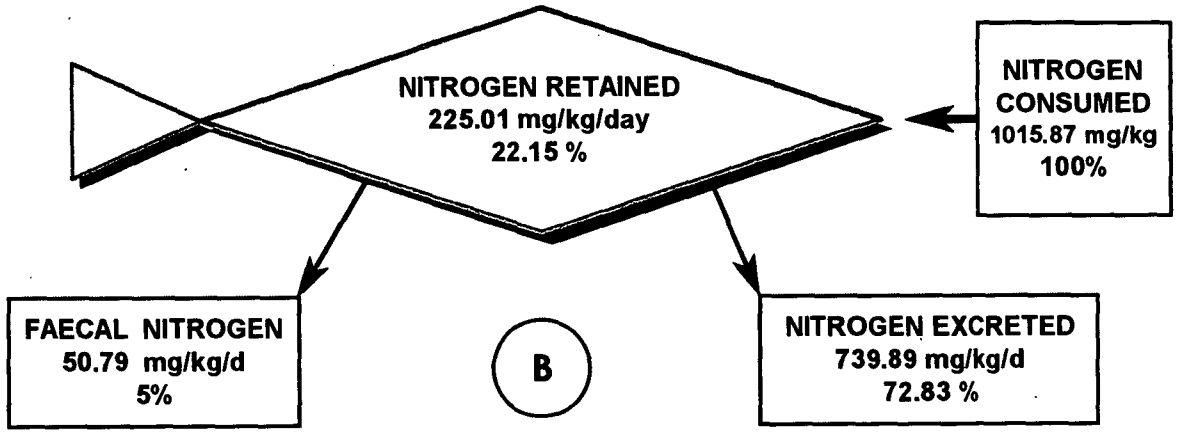
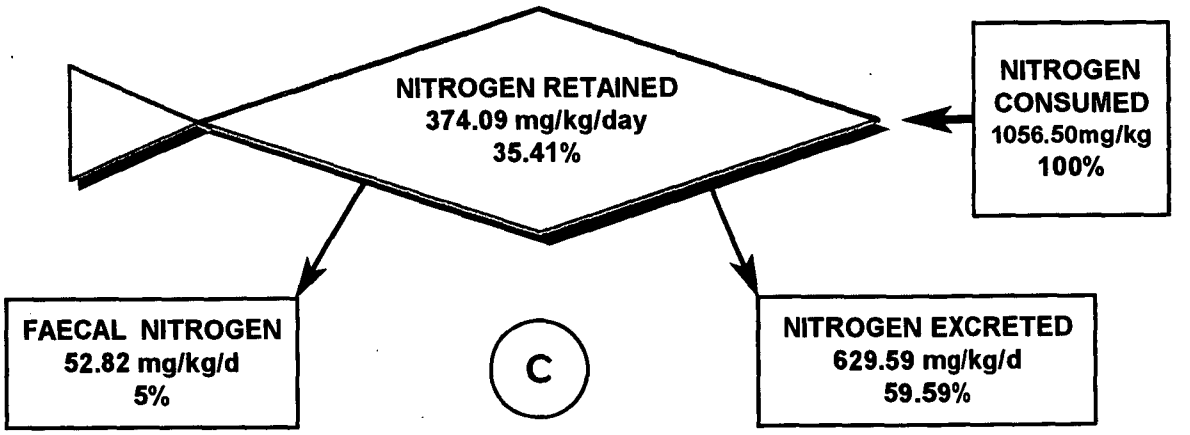
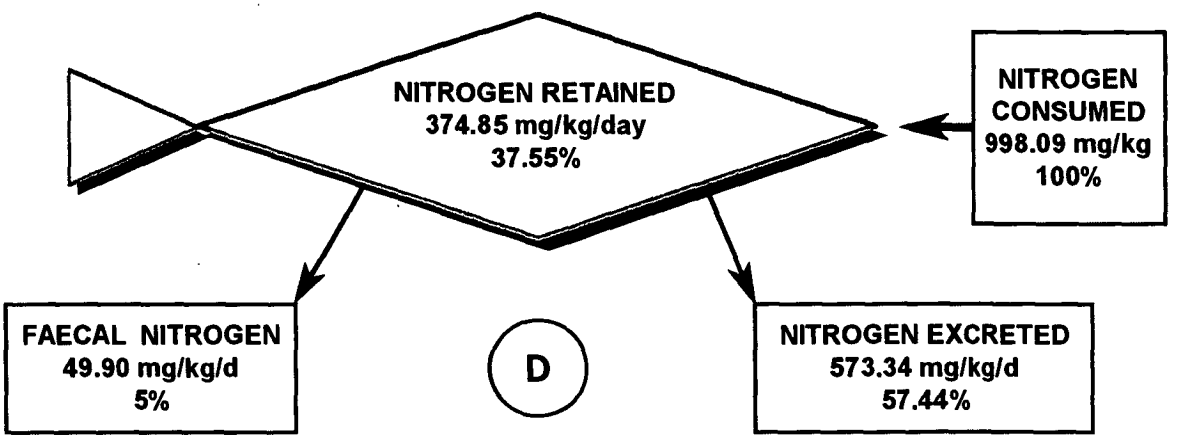

Nitrogen Retained, mg N kg -1 day-1
450
400
350
300
250
200
150
100
50
0
-50
-100
-150
-200
-250
-300
-350
300
600
900
Nitrogen Consumed, mg N kg -1 day -1

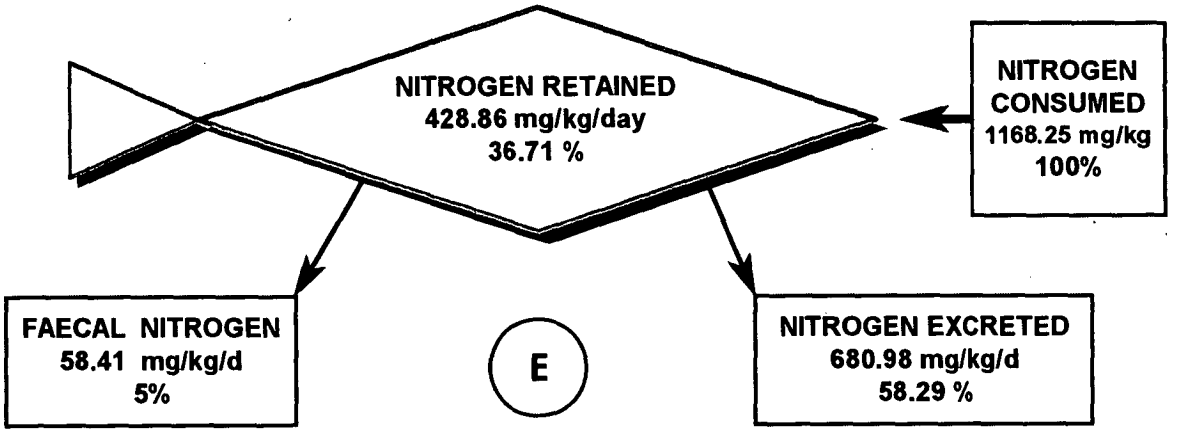
Figure 4.2. Protein-Nitrgen Flux in juvenile greenback flounder (approx. 1g) fed diets containing (a) 30% (b) 35% (c) 40% (d) 45% (e)50% and (f) 55% protein.

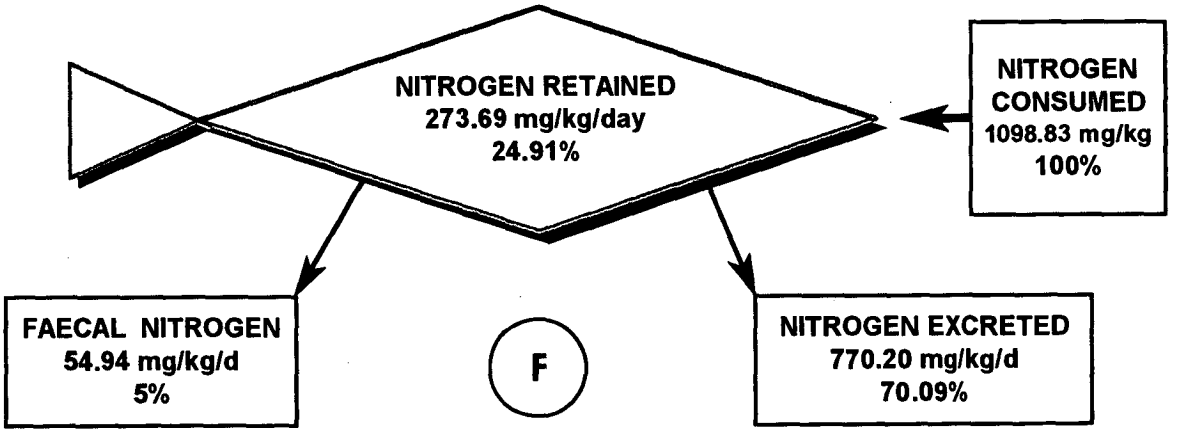

NITROGEN RETAINED
225.18 mg/kg/day
24.70%
NITROGEN CONSUMED
911.74 mg/kg
100%
FAECAL NITROGEN
45.58 mg/kg/d
5%
A
NITROGEN EXCRETED
640.98 mg/kg/d
70.30%


NITROGEN RETAINED
225.01 mg/kg/day
22.15 %
NITROGEN CONSUMED
1015.87 mg/kg
100%
FAECAL NITROGEN
50.79 mg/kg/d
5%
B
NITROGEN EXCRETED
739.89 mg/kg/d
72.83 %


NITROGEN RETAINED
374.09 mg/kg/day
35.41%
NITROGEN CONSUMED
1056.50mg/kg
100%
FAECAL NITROGEN
52.82 mg/kg/d
5%
C
NITROGEN EXCRETED
629.59 mg/kg/d
59.59%


NITROGEN RETAINED
374.85 mg/kg/day
37.55%
NITROGEN CONSUMED
998.09 mg/kg
100%
FAECAL NITROGEN
49.90 mg/kg/d
5%
D
NITROGEN EXCRETED
573.34 mg/kg/d
57.44%


NITROGEN RETAINED
428.86 mg/kg/day
36.71 %
NITROGEN CONSUMED
1168.25 mg/kg
100%
FAECAL NITROGEN
58.41 mg/kg/d
5%
E
NITROGEN EXCRETED
680.98 mg/kg/d
58.29 %


NITROGEN RETAINED
273.69 mg/kg/day
24.91%
NITROGEN CONSUMED
1098.83 mg/kg
100%
FAECAL NITROGEN
54.94 mg/kg/d
5%
F
NITROGEN EXCRETED
770.20 mg/kg/d
70.09%

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