The impact of lysine and arginine ratios in plant-based protein diets on appetite, growth performance and gene expression of brain neuropeptide Y (NPY) and cholecystokinin (CCK) in juvenile cobia (*Rachycentron canadum*)

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Dissertation for the degree of philosophiae doctor (PhD)

University of Bergen

December 2012

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This study was funded by The Norwegian Agency for Development Cooperation through the SRV-2701 project: 'Improving Training and Research Capacity of Nha Trang University, Viet Nam-Phage 2". Experiments from the present study were carried out at the Faculty of Aquaculture, Nha Trang University (formerly named University of Fisheries)-Vietnam from June 2009 to January 2011. The study was then continued at the Department of Biology, University of Bergen, and the National Institute of Nutrition and Seafood Research (NIFES) under the continued supervision of Professor Ivar Rønnestad and Dr. Marit Espe.

ACKNOWLEDGEMENTS

First of all, I wish to thank my main supervisor, Professor Ivar Rønnestad, who gave me excellent supervision and continuous support from the beginning to the end of my study. I would also like to thank my co-supervisor, Dr. Marit Espe, who gave me invaluable supervision and immediately improved the text in numerous draft versions of the manuscript whenever she touched it. I really appreciate Prof. Ivar Rønnestad and Dr. Marit Espe for sharing me the vast wisdom and intellectual ideas in the field of science and every aspect of life. This dissertation would never have been finished without the guidance, support and criticism of my supervisors throughout numerous draft versions of the manuscript. Sincerely thanks to my co-supervisor, Associate Professor Lai Van Hung for his guidance and support during my study.

Thanks to the leaders of the project SRV2701 and Nha Trang University, Rector Dr. Vu Van Xung, Vice-Rector Assoc. Prof. Trang Si Trung, and Dean Dr. Pham Quoc Hung, and accountant Ngo Kim Thoa for their support during my study.

I would like to express my gratitude to Dr. Louise Buttle from EWOS Innovation As, Norway for producing the test diets for the experiments of the study and for her valuable comments on the thesis manuscript.

I would like to thank the University of Bergen (UiB), Nha Trang University (NTU) and the National Institute of Nutrition and Seafood Research (NIFES) for providing me excellent academic milieu with equipped laboratory facilities. Thanks to EWOS Innovation for proving facilities to extrude the test diets.

Thanks also to Vo Ngoc Tham and Ngo Anh Tuan at the Faculty of Aquaculture, NTU, for their support and encouragement. I would like to thank Dr. Ann-Elise Olderbakk Jordal and Tharmini Kalananthan at UiB for providing invaluable help, criticism and technical guidance. Thanks to Margrethe Rygg, Anita Birkenes, Kari Sæle and Martin Joseph Malaiamaan at NIFES for invaluable technical advice. I am really grateful to my colleagues at NTU, Dang Thuy Binh, Ho Manh Tuan, Hoang Van Dan, Pham Thi Anh, Luong Thi Hau, Pham Thi Khanh and Banh Thi Quyen Quyen for their assistance in sampling and taking care of the fish.

I would like to extend a special thank to Professor Knut Heen (Univ of Tromsø) SRV 2701 project adviser, Professor Heidrun Inger Wergeland, Sidsel Kjølleberg, Berit Øglænd, Assoc.-

-Prof. Glenn Bristow and Tommy Strand at the University of Bergen for support and encouragement during my study. I am really grateful to Prof. Geir Kåre Totland, Prof. Jon Vidar Helvik and Rita Karlsen, and all other members at the Marine Developmental Biology, UiB, for making life in Bergen more interesting. Special thanks also to all members from my friends' families, Nguyen Quang Phuc, Anh Duc, Anh Huy, Mach Thi Ngoc Diep for their encouragement and friendship.

Finally, I would like to thank you very much my wife, Tran Thi Bao Tien and my three year-old son, Nguyen Anh Kiet, and my parents, Nguyen Tan Khoa and Nguyen Thi Chap and my father in law, Tran Cong Khe for their love, support and encouragement during my study.

Bergen, December 2012

Nguyen Van Minh

CONTENTS

ABSTRACT

Aquaculture of cobia, *Rachycentron canadum* is hampered by lack of good feeding protocols and nutritionally optimized diets. Studies on the role of appetite and feeding behavior regulating neuropeptides in cobia have not been pursued to date. The current study initially assessed the impact of plant-based protein diets with different lysine (L) to arginine (A) ratios on appetite and feed intake, feed efficiencies, growth performance, and the deposition of protein and lipid in juvenile cobia. In addition, this study also aimed to determine whether there is a link between the dietary ratio of lysine to arginine, feed consumption, and expression of cobia brain neuropeptides (*npy* and *cck*).

In a pilot experiment, juvenile cobia were fed to satiety with two commercial diets (CD1 and CD2) or the plant-based protein test diet with balanced lysine to arginine ratio (1.1; BL/A) had equal feeding rate of 5.3-5.4±0.3% BW (for a meal). No differences in stomach filling occurred between cobia fed the test diet and the two commercial diets. Gastric evacuation rates in cobia were performed as an exponential relationship, and were estimated as the function $Y_T=V_0\, e^{-b(x)}$ (V_T, volume of feed at time T; V_0, volume of feed at time 0; b, the instantaneous evacuation rate; and x, time postprandial; $R^2>0.95$). Between 77 to 80% of the stomach contents were evacuated to the lower parts of the gastrointestinal tract at 8 h, and most of consumed feed (98%) was emptied out of the stomach at 16 h postprandial.

In experiment I, juvenile cobia fed with two commercial diets (CD1 and CD2) and the BL/A diet (for 4 weeks) grew as well as one of the commercial diet, but better than the other. However, cobia fed the CD2 diet deposited more lipid than cobia fed the BL/A diet. Cobia fed the BL/A diet obtained better weight gain, feed conversion ratio and protein gain than cobia fed the commercial diet CD1, while lipid gain was less in fish fed the BL/A diet. No differences in plasma amino acid profile analyzed at 24 h postprandial were observed between cobia fed any of the three diets. Thus, juvenile cobia had the ability to grow and utilize the plant-based protein diet and these two commercial diets.

In experiment II, juvenile cobia were fed to satiety with three test diets of plant-based protein and different lysine to arginine ratio (0.8, LL/A; 1.1, BL/A; and 1.8, HL/A) diets and the CD2 diet as a control for growth for 6 weeks. Similar growth performance was found for the balanced lysine to arginine ratio diet, but imbalanced lysine to arginine ratio reduced performance.

In experiment III, juvenile cobia were fed to satiety with the LL/A-, BL/A- and HL/A diet, using the CD2 diet as control for 6 weeks. Periprandial expression of brain mRNA for NPY and CCK (*npy* and *cck*) were measured twice, at weeks 1 and 6. Feed intake and growth performance were also recorded. At week one, levels of expressed brain *npy* were significantly higher in pre-feeding cobia (unfed) than fed cobia, except in the fish fed the LL/A diet. At week six, expression of brain *npy* in unfed cobia was significantly higher than in fed cobia for all diets. For brain *cck*, there were no significant differences in expression, regardless of feeding status (pre-feeding or after a meal with any of the diets) or time point. This indicates that NPY serves as an orexigenic factor in cobia. No clear correlations between the dietary lysine to arginine ratios and feed intake, growth and expression of *npy* and *cck* was found. Cobia fed the LL/A diet had lower feed intake than those fed the BL/A- and control diet, while cobia fed both imbalanced diets (LL/A and HL/A) had reduced growth, compared to cobia fed the BL/A- and control diet. This suggests the existence of a mechanism that links dietary lysine and/or arginine to regulation of appetite and feed intake in cobia.

In conclusion, cobia grew and utilized the diet with high plant ingredient inclusion. Imbalanced dietary lysine to arginine ratios negatively affected feed intake and feed efficiencies, thus reduced growth. Brain *npy* levels in fed cobia were significantly lower than that in pre-feeding cobia, suggesting that NPY is an orexigenic factor in cobia. Dietary lysine to arginine affected the expression of *npy* at short-term feeding, but not at long-term feeding, suggested that feeding-regulating adaptation may exist in cobia. Meanwhile, brain *cck* levels in unfed cobia did not differ from that in fed cobia, regardless of dietary lysine to arginine ratios or time point in the current study.

LIST OF PAPERS

This thesis is based on the following papers:

Paper I

Minh Van Nguyen, Ivar Rønnestad, Louise Buttle, Hung Van Lai and Marit Espe

Evaluation of a high plant protein test diet for juvenile cobia (*Rachycentron canadum*) shows growth comparable to commercial diets. (Submitted to Fisheries Science)

Paper II

Minh Van Nguyen, Ivar Rønnestad, Louise Buttle, Hung Van Lai and Marit Espe

Imbalanced lysine to arginine ratios reduced performance in juvenile cobia (*Rachycentron canadum*) fed high plant protein diets. (Submitted to Aquaculture Nutrition)

Paper III

Minh Van Nguyen, Ann-Elise Olderbakk Jordal, Marit Espe, Louise Buttle , Hung Van Lai and Ivar Rønnestad.

Feed intake and brain neuropeptide Y (NPY) and cholecystokinin (CCK) gene expression in juvenile cobia fed plant-based protein diets with different lysine to arginine ratios. (Submitted to Comparative Biochemistry and Physiology - Part A)

Of which will be referred as paper I, II and III in the text

LIST OF FIGURES

LIST OF ABBREVIATIONS

PE Pilot experiment
L Lysine
A Arginine
L/A Lysine to arginine ratio
LL/A Low lysine to arginine ratio (Lys/Arg; 0.8)
BL/A Balanced lysine to arginine ratio (Lys/Arg; 1.1)
HL/A High lysine to arginine ratio (Lys/Arg; 1.8)
h Hour
AA Amino acid
DAA Dispensable amino acid
IAA Indispensable amino acid
FAA Free amino acid
HPV Hepatic portal vein
DA Dorsal aorta
FCR Feed conversion ratio
FI Feed intake
HSI Hepatosomatic index
VSI Viscerosomatic index
PPV Protein productive value
PER Protein efficiency ratio
WG Weight gain
BW Body weight
NPY Neuropeptide Y
npy Gene/nucleotide sequence encoding NPY (or NPY mRNA)
CCK Cholecystokinin
cck Gene/nucleotide sequence encoding CCK (or CCK mRNA)
mRNA Messenger RNA
PCR Polymerase chain reaction
Q-PCR Quantitative PCR
MNE Mean normalized expression
SEM Standard error of the mean

1. GENERAL INTRODUCTION AND BACKGROUND

1.1 Cobia and aquaculture

Cobia, *Rachycentron canadum* Linnaeus (1766), is the only species of the family Rachycentridae (FishBase 2012), and is widely distributed in subtropical, tropical and temperate areas, except for the central and eastern Pacific (Briggs 1960). Cobia is also known/referred to as ling, lemon fish and black kingfish. Cobia is a perciform, pelagic and carnivorous marine fish that feeds on crustaceans, small fish and cephalopods (Arendt *et al.* 2001, Franks *et al.* 1996). In the wild cobia can grow up to 68 kg in body weight, and 2 m in length, and has a long life span up to 15 years (Kaiser and Holt 2005, Wheeler 1975).

Cobia has many favorable production- related characteristics, such as rapid growth, and thus is regarded as a good candidate species for aquaculture. Under optimal feed and temperature condition cobia fingerlings can reach the marketable size of 4-6 kg (Chou *et al.* 2001) or even 6-10 kg (Su *et al.* 2000) within a year. Additionally, cobia is highly marketable prized because the fillet of the fish is lean, and of high quality with white, firm and good flavored flesh that is also suitable for the sashimi industry (Chou *et al.* 2001). Study on cobia off the coast of North Carolina in the early 1970s showed that the fish had fast growth rate and broad distribution, and therefore a high potential to aquaculture (Hassler and Rainville 1975). Induced spawning in brood-stock cobia can be achieved by hormone injections (Franks *et al.* 2001), ambient seasonal cycles (Arnold *et al.* 2002, Liao *et al.* 2004), and photo-thermal conditioning (Kaiser and Holt 2004). Hatchery production of cobia juveniles for offshore grow-out cage culture shows promising results (Benetti *et al.* 2008, Holt *et al.* 2007, Liao *et al.* 2004). Further, cobia is able to adapt to a wide range of salinity (Faulk and Holt 2006, Resley *et al.* 2006), and shows a positive response to vaccination (Lin *et al.* 2006). Due to these desirable characteristics coupled with the success of commercial cobia production since the late 1990s, aquaculture of cobia has risen dramatically in Taiwan and the USA (Benetti *et al.* 2008, Liao *et al.* 2004). Cobia farming also has started in China, the Philippines, the EU, Brazil, Panama and Vietnam (Holt *et al.* 2007, Nhu *et al.* 2011). However, as cobia recently has been introduced into aquaculture, documentation on the nutritional and amino requirements in this species is still limited (Fraser and Davies 2009, Holt *et al.* 2007). Aquaculture of cobia is still hampered by lack of good feeding protocols and nutritionally optimized diets.

Some initial studies of requirements for protein and lipid, and for some indispensable amino acids have been conducted on cobia. According to Chou and coworkers, a crude protein

concentration of 445 g kg^{-1} dry matter diet would give maximum growth in cobia, while optimum dietary lipid for juvenile cobia was found to be 57.6 g kg^{-1} dry matter (Chou *et al.* 2001). Juvenile cobia fed 400 g kg^{-1} dietary crude protein showed better feed efficiencies than those fed diet containing 500 g kg^{-1} crude protein; while dietary lipid levels (60, 120 and 180 g kg^{-1} diet) did not affect weight gain or feed utilization (Craig *et al.* 2006). Methionine and lysine requirement, is established for cobia to be 11.9 g methionine in the presence of 6.7 g cysteine kg^{-1} dry matter and 23.3 g lysine kg^{-1} dry matter, respectively (Zhou *et al.* 2006, Zhou *et al.* 2007). Lunger and coworkers reported that taurine should be supplemented at 5 g taurine kg^{-1} dry matter when fishmeal were replaced by yeast based proteins in juvenile cobia to maximize weight gain and feed efficiency (Lunger *et al.* 2007a).

1.2 Replacement of fishmeal by plant proteins in aquafeeds

One of the greatest concerns that have been targeted by aquafeed industry is the replacement of fishmeal by other alternative dietary proteins, for example plant protein sources (soy concentrates and corn gluten, etc.). These alternative sources of protein are considered more sustainable, compared to fishmeal (FAO 2009, Gatlin *et al.* 2007) that has traditionally constituted the protein source in fish feed (Miles and Chapman 2006). Nevertheless, plant-derived nutrient ingredients contain broad range of anti-nutritional substances depending on the source. Thus, inclusion of plant ingredients in the diet may lead to poor palatability, reduced palatability and/or digestibility, that consequently reduce growth performance in fish (Cho *et al.* 1974, Dabrowski *et al.* 1989, Olli *et al.* 1994, Rumsey *et al.* 1994, Spinelli *et al.* 1983), and crustaceans (summarized by Venero *et al.* 2008). In addition, reduced digestibility may cause environmental problems due to increased indigestible wastes. Anti-nutritional substances that may be present in plant protein ingredients affect protein utilization and digestion, such as protease (trypsin) inhibitors, tannins and lectins (El-Sayed 1999, Murai 1992). Further, plant-derived nutrient sources may contain phytates, gossypol pigments, oxalates and glucosinolates or antivitaimins that influence mineral utilization as well as affect the availability and functioning of vitamins (Francis *et al.* 2001). In addition, plant-derived nutrient sources also may contain miscellaneous substances such as mycotoxins, mimosine, cyanogens, nitrate, alkaloids, photosensitizing agents, phytooestrogens and saponins (El-Sayed 1999, Francis *et al.* 2001). Most of these above anti-nutritional factors, however, can be eliminated through processing techniques, for example dry or wet heating, rolling into flakes, de-oiled by a solvent, and toasted. Although, thermal treatment during processing may alter the chemical nature and reduce the nutritional quality of proteins and carbohydrates (Francis *et al.* 2001).

With the improvement of plant product processing techniques and better understanding of the nutritional requirements, replacement of fishmeal by plant protein sources in aquaculture diets has showed promising results (Berge *et al.* 1999b, Carter and Hauler 2000, Day and Gonzalez 2000, Espe *et al.* 2006, Mundheim *et al.* 2004). However, different species show different response to the inclusion of plant ingredients in the diet. Up to 660 g kg^{-1} of fishmeal could be replaced by blends of vegetable proteins without negative effects on growth performance of rainbow trout, *Oncorhynchus mykiss* (Gomes *et al.* 1995). Replacement of up to 600 g kg^{-1} of fishmeal by corn gluten meal did not affect growth or feed efficiency in gilthead seabream, *Sparus aurata* (Pereira and Oliva-Teles 2003), while a combination of corn gluten meal, soy protein concentrate and wheat gluten successfully replaced all dietary fishmeal for the species (Kissil and Lupatsch 2004). Khan *et al.* (2003) reported that soybean meal, supplemented with methionine and fortified with minerals, could totally replace fishmeal in diets without affecting growth and feed utilization in rohu, *Labeo rohita*. Atlantic halibut, *Hippoglossus hippoglossus*, tolerated a replacement of up to 300 g kg^{-1} fishmeal with wheat gluten (Helland and Grisdale-Helland 2006, Murray *et al.* 2010) or 360 g kg^{-1} replacement with full-fat soybean without any negative effect on growth rate, feed intake, feed efficiency or protein retention (Grisdale-Helland *et al.* 2002). In cobia, up to 400 g kg^{-1} of fishmeal can be replaced with soybean meal without negatively affect growth and feed conversion ratios (Chou *et al.* 2004, Zhou *et al.* 2005). While, Atlantic salmon fed diets devoid of fishmeal with added crystalline amino acids to mimic amino acids profiles of fishmeal did not differ from the fish fed fishmeal- based diets in feed conversion ratios and protein accretion (Espe *et al.* 2006). A total replacement of fishmeal with rapeseed protein concentrate did not cause any negative effects on feed intake and feed efficiency, and growth or intestinal morphological deficiencies in rainbow trout, as compared to the fish fed fishmeal-based diet; suggesting a great potential of using this source of protein in formulation for rainbow trout (Slawski *et al.* 2012).

Another factor that needs to be taken into account when fishmeal protein is replaced by plant protein ingredients in aquafeeds is that plant proteins may not have a well balanced profile for indispensable amino acids (Venero *et al.* 2008). Therefore, high plant protein inclusion in the diet often results in reduced growth and feed intake in fish (de Francesco *et al.* 2004, Gomes *et al.* 1995). In order to maximize growth and feed utilization in fish fed plant-based protein feed, a blend of plant protein ingredients is formulated in combination with supplementation of crystalline amino acids. By doing so, dietary amino acid profiles fulfill the requirement and/or mimic the amino acid profiles of the fishmeal-based diets (Cheng *et al.* 2003, Chou *et al.* 2004, Espe *et al.* 2006; 2007; 2012a; 2012b, Gallagher 1994, Sveier *et al.* 2001). Though, the

understanding of nutrient requirements for indispensable amino acids in fish is still limited and is available for a few species only (NRC 2011).

1.3 Lysine and arginine

Lysine and arginine are indispensable amino acids for fish, and are often present in low concentrations in gluten or corn-based proteins and in casein (Ball *et al.* 2007, Espe *et al.* 2007, Mai *et al.* 2006, Small and Soares 2000, Wu *et al.* 2009). In addition to protein turnover, lysine and arginine are involved in a range of metabolic and physiological functions. Lysine is the precursor for carnitine, which is required for the transport of long-chained fatty acids from the cytosol into mitochondria for β-oxidation and subsequent energy production (Harpaz 2005, Walton *et al.* 1984). Further, lysine also affects collagen synthesis, as its hydroxylation product, hydroxylysine, is necessary for formation of the intermolecular crosslinks in collagen (Eyre 1980, Piez and Likins 1957). Fig. 1 summarizes metabolism pathways of lysine in fish.

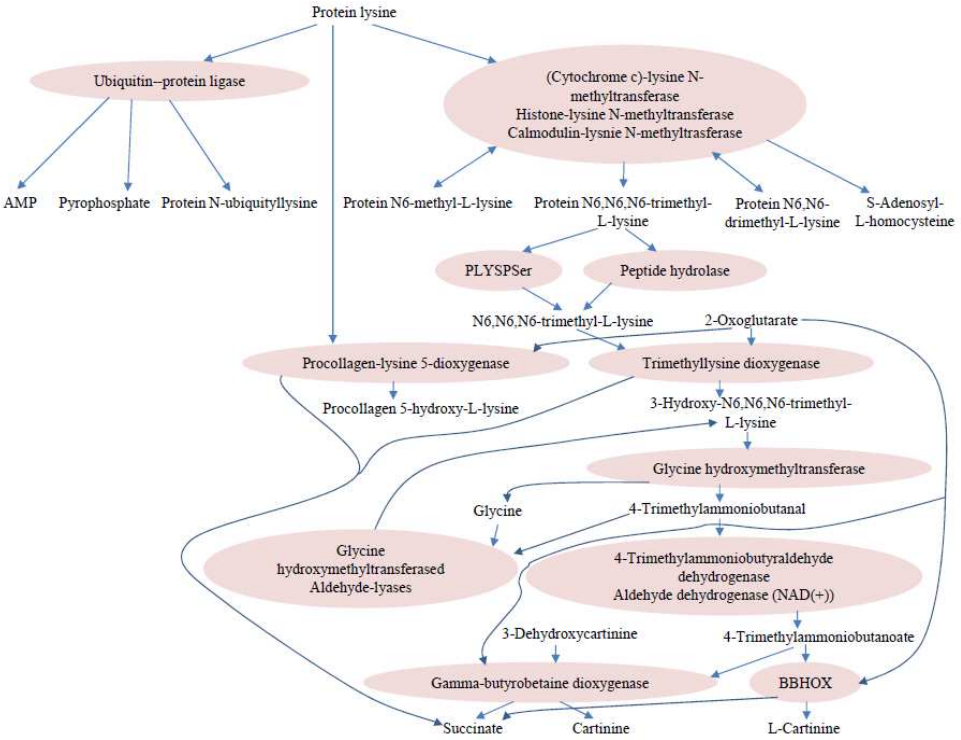

Fig. 1. Summary of lysine metabolism in fish (Adapted from MetaFishNet 2012). PLYSPSer, protein lysine peptidase (endoplasmic reticulum); $NAD(^{+})$, Nicotinamide adenine dinucleotide; BBHOX, 4-Trimethylammoniobutanoate,2-oxoglutarate oxygenoxidoreductase (3-hydroxylating); (For simplification, several substrates and enzymes involved in the metabolic pathways are not shown).

Arginine is the precursor for synthesis of nitric oxide, urea, polyamines, proline, glutamate, creatine and/or agmatine (Hird 1986, Wu and Morris 1998). Arginine is involved in endocrine regulation, such as being a stimulator of insulin and IGF-1 (Banos *et al.* 1999, Plisetskaya *et al.* 1991). Further, arginine participates in the regulation of extra-endocrine signaling pathways including AMP-activated protein kinase (AMPK) and the target of rapamycin, TOR (Jonsson *et al.* 2006, Yao *et al.* 2008). Arginine is also known to affect immune functions (Li *et al.* 2007, Wu *et al.* 2004), as well as reproductive performance in mammals (Mateo *et al.* 2007). Metabolic pathways of arginine are depicted in the following figure (Fig. 2).

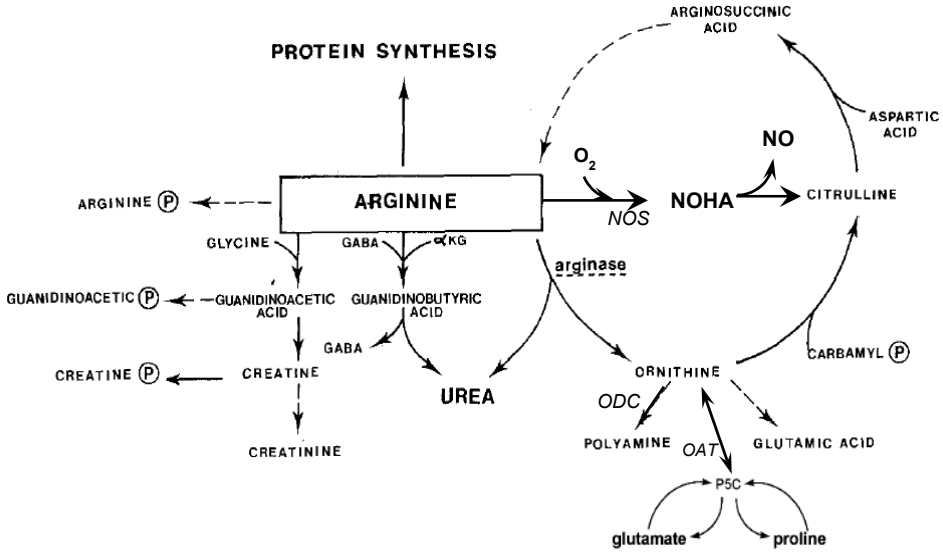

Fig. 2. Schematic representation of the different pathways of arginine metabolism. P5C, L-Δ^1-pyrroline-5-carboxylate; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase; GABA, γ- aminobutyric acid; αKG, α- ketoglutanate; NO, nitric oxide; NOS, NO synthase; NOHA, N^G-Hydroxy-L-arginine. Broken arrows indicate pathways demonstrated in invertebrates or in mammals but not in fish (Adapted from Kaushik *et al.* (1988) and Morris (2007), with modifications).

Deficiency of lysine has been reported to cause loss of appetite, and reduced growth performance in milkfish, *Chanos chanos*, Indian major carp, *Cirrhinus mrigala*, and Japanese seabass, *Lateolabrax japonicus* (Ahmed and Khan 2004b, Borlongan and Benitez 1990, Mai *et al.* 2006), as well as increased mortality, and incidence of erosion of dorsal and caudal fin in rainbow trout (Ketola 1983, Walton *et al.* 1984). However, excessive lysine levels in the diet did not improve growth performance and feed utilization in yellowtail, *Seriola quinqueradiata*,

seabream, *Pagrus major*, grouper, *Epinephelus coioides*, cobia and Atlantic salmon, *Salmo salar* (Forster and Ogata 1998, Grisdale-Helland *et al.* 2011, Luo *et al.* 2006, Ruchimat *et al.* 1997, Zhou *et al.* 2007). Dietary exccessive lysine causes negative impacts on growth performance and feed efficiencies in various fish species, for instance, in rainbow trout, Indian major carp, grass carp, *Ctenopharyngodon idella*, Japanese seabass, gilthead seabream, pacu, *Piaractus mesopotamicus*, and Pacific threadfin, *Polydactylus sexfilis* (Ahmed and Khan 2004b, Bicudo *et al.* 2009, Deng *et al.* 2010, Mai *et al.* 2006, Marcouli *et al.* 2006, Walton *et al.* 1984, Wang *et al.* 2005).

Deficiency of arginine reduced growth and feed efficiencies in European seabass, *Dicentrarchus labrax*, coho salmon, *O. kisutch* and rohu, *Labeo rohita* (Abidi and Khan 2009, Luzzana *et al.* 1998, Tibaldi *et al.* 1994), as well as increased mortality and the incidence of lordosis in common carp, *Cyprinus carpio* (Tacon 1992). However, feeding diets of higher concentration of arginine than the requirements may cause reduction on weight gain and feed efficiencies in rainbow trout, Nile tilapia, *Oreochromis niloticus,* Indian major carp, rohu, and hybrid clarias, *Clarias garienpinus x C. marcrocephalus* (Abidi and Khan 2009, Ahmed and Khan 2004a, Kaushik and Luquet 1980, Santiago and Lovell 1988, Singh and Khan 2007), as well as reduced protein efficiency ratio in Indian major carp (Abidi and Khan 2009).

Lysine and arginine are assumed to share and/or compete for the same trans-membrane carrier systems. There are several transporters described that handle these cationic amino acids (Narawane 2011) at both intestinal and renal level (Closs *et al.* 2004, Cynober *et al.* 1995). The metabolism and utilization of one of the amino acid affects the other and may give negative effects on growth (Bedford *et al.* 1987, Berge *et al.* 2002, Fico *et al.* 1982, Harper *et al.* 1970, Jones *et al.* 1967, Nesheim 1968). The interactions between lysine and arginine absorption, and thus availability of these amino acids within body compartments, cause reduced growth in various mammalian species (Anderson *et al.* 1979, Czarnecki *et al.* 1985, Jones *et al.* 1966), and teleosts (Berge *et al.* 2002, Borlongan 1991, Davies *et al.* 1997, Santiago and Lovell 1988, Zhou *et al.* 2011a). However, complete understanding of absorption, metabolism and utilization of lysine and arginine in cobia is still limited. Whether there are individual or interactive effects between the two amino acids that directly or indirectly affect feeding behavior and appetite in cobia remains to be investigated.

1.4 Appetite and feed intake and neuropeptide Y and cholecystokinin

Appetite and feed intake

Feeding behavior and appetite show large differences between fish species and also vary with a range of factors including developmental stage, physiological and health status (stress or disease), season and feeding status (Dabrowski 1983, Fletcher 1984, Talbot *et al.* 1984, Volkoff *et al.* 2010). In addition, quality of the feed (nutrient composition, food size or palatability) and environmental conditions (temperature, oxygen, light) also influence the feeding behavior (Volkoff *et al.* 2010). It is obvious that fish will not grow well if they have not consumed adequate amounts of food. In most cases, marine fish show reduced growth when they are offered diets with high inclusion of alternative protein ingredients, as mentioned above, is as a consequence of reduced appetite, feed intake and/or low palatability of such diets. In aquaculture, feeding the fish with diets of low palatability and low digestibility will result in reduced feed intake, feed efficiencies and growth performance. Therefore, this is a not a cost-effective feeding technique. In addition, if the fish show low appetite and less food consumption, more food will be lost in the water column, especially in auto feeding practice systems. Not only does this increase fish production cost, but also causes more environmental pollution due to more uneaten feed released into the water. Thus, understanding the mechanism regulating feeding behavior and appetite and nutrient metabolism is essential as it helps to formulate a better diet that provides good feeding efficiency and growth in fish.

Feeding behavior and appetite in fish are regulated in the brain by a variety of orexigenic (appetite-stimulating) and anorexigenic (appetite-inhibiting) factors. The regulatory pathways receive input not only from the central nervous system, but also from the gastro-intestinal tract and peripheral tissues like the adipose tissues (Ueta *et al.* 2003, Volkoff *et al.* 2005). Table 1 summarizes orexigenic and anorexigenic factors that are involved in the regulation of feeding in mammals. Several peptides homologous to the mammalian appetite-regulating peptides have been characterized in fish (for a review, see Volkoff *et al.* 2010). It appears that the same series of neuropeptides and their receptor systems are involved in the control of feeding behavior in fish as in mammals, although the mode of action of neuropeptides and their receptor systems in fish may differ from that in mammals (Matsuda 2009). Some of the neuropeptides that are involved in feed intake regulation in fish are depicted in Fig. 3. Neuropeptide Y (NPY) and cholecystokinine (CCK) are among the neuropeptides that affect feeding behavior as well as appetite in both mammals and teleosts (Crawley and Corwin 1994, Lin *et al.* 2000, Matsuda *et al.* 2012, Mercer *et al.* 2011, Rehfeld 2004).

Neuropeptide Y (NPY)

NPY is a 36-amino acid peptide that was first isolated from porcine brain (Tatemoto 1982). NPY belongs to the pancreatic polypeptide family that includes NPY, peptide YY, pancreatic polypeptide and peptide Y (Matsuda *et al.* 2012, Pedrazzini *et al.* 2003, Volkoff *et al.* 2005). NPY and its related peptides as well as their receptors (Y1-Y6) are highly conserved neuroendocrine peptides in vertebrates through evolution (Blomqvist *et al.* 1992, Matsuda *et al.* 2012). Previous studies in mammals reported that NPY is involved and integrated in several endocrine signaling pathways, such as appetite and feed intake regulation, energy homeostasis, vasoconstriction, circadian rhythmicity and pituitary hormone release (Mercer *et al.* 2011, Pedrazzini *et al.* 2003). In fish, NPY is also known to be involved in the regulation of feeding behavior and psychomotor activity and the pituitary hormone release (Matsuda *et al.* 2012, Wong *et al*. 2006).

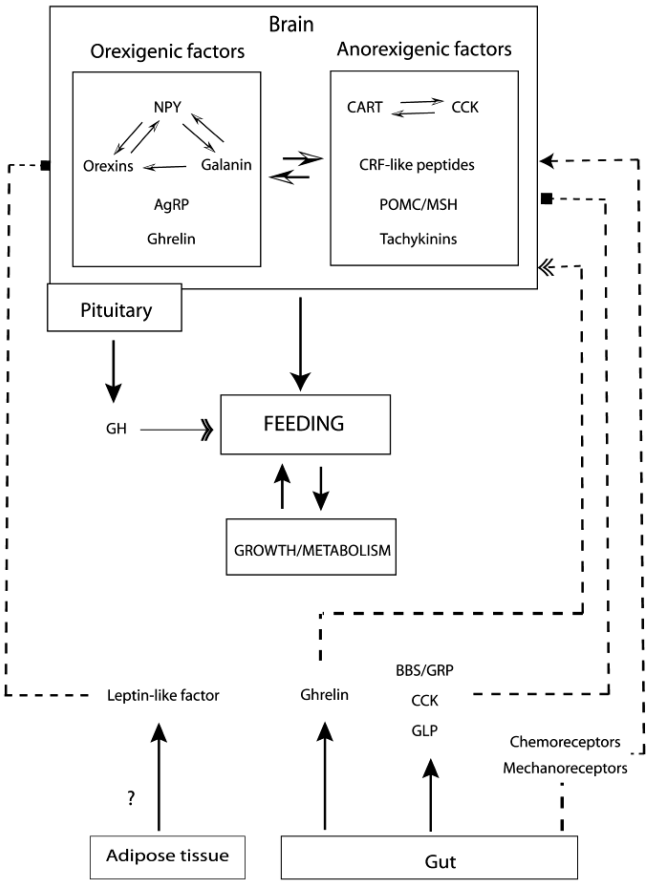

Fig. 3. Schematic diagram of some of the central and peripheral peptides known to regulate food intake in fish. Within the brain, central neuropeptide systems receive signals and integrate information from the periphery to adjust food intake. These central systems consist of both feeding stimulators (orexigenic factors) and feeding inhibitors (anorexigenic factors). Some of these neuropeptide systems interact with each other. These interactions are indicated by arrows. Peripheral hormonal signals from gut and possibly from adipose tissue reach the brain and influence the central neuropeptide signals. Peripheral signals are conveyed via sensory axons in the vagus nerve or reach the brain directly via the blood. Double arrowheads indicate an orexigenic action whereas square arrowheads indicate an anorexigenic action. AgRP, agouti-related protein; BBS/GRP, bombesin/gastrin-releasing peptide; CART, cocaine- and amphetamine-regulated transcript; CCK, cholecystokinin; CRF, corticotropin-releasing factor; GH, growth hormone; GLP, glucagon-like peptide; MSH, melanocyte-stimulating hormone; NPY, neuropeptide Y; and POMC, proopiomelanocortin (Adapted from Volkoff *et al*. 2005).

NPY is well known to function as a hunger stimulus that is involved in regulation of feed intake in mammals (Mercer *et al.* 2011) and fish (Volkoff *et al.* 2005). Central injections of NPY result in induced feed intake in a dose-dependent manner in mammals (Clark *et al.* 1985, Levine and Morley 1984) and fish (Aldegunde and Mancebo 2006, Lopez-Patino *et al.* 1999, Narnaware *et al.* 2000, Silverstein and Plisetskaya 2000). Previous studies have shown elevated levels of expression of *npy* (encoding gene of NPY; i.e. mRNA) in the brain of fish in connection to feed intake in cartilaginous fish and teleosts. Goldfish, *Carassius auratus* showed increased brain *npy* at 1-3 h before the mealtime, suggesting the involvement of NPY in the signal pathways that control feeding behavior in the fish (Narnaware *et al.* 2000). Brain *npy* in Atlantic cod, *Gardus morhua* was higher at the mealtime (0 h) than 2 h before the meal (-2 h) (Kehoe and Volkoff 2007). Similar results were reported in Brazilian flounder, *Paralichthys orbignyanus* that brain *npy* levels were higher at the start of a meal (0 h) compared to 2 h before the mealtime (-2) and 2 h after feed intake (Campos *et al.* 2012). Food deprivation also affects expression of *npy*, and an increase in brain *npy* expression was reported during fasting in coho salmon, chinook salmon, *O. tshawytscha*, goldfish and winter skate, *Raja ocellata* (MacDonald and Volkoff 2009, Narnaware and Peter 2001, Silverstein *et al.* 1998).

Cholecystokinin (CCK)

CCK is a neuropeptide that is synthesized from prepro-CCK and through posttranslational specific site-cleaving gives rise to CCK-83, -58, -33, -22 and 8 (Eberlein *et al.* 1992). All CCK forms share the common C-terminus of Trp-Met-Asp-Phe-NH_2 with their peptide-family member, gastrin (Crawley and Corwin 1994, Volkoff *et al.* 2005). CCK has a variety of biological functions in addition to its role in appetite regulation, that include stimulation of gall bladder contraction, stimulation of pancreatic enzyme release, slowing down the gastric emptying, interactions with dopamine in the mesolimbic pathway, and induction of anxiety-like behaviors (Crawley and Corwin 1994, Rehfeld 2004, Rojas-Garcia *et al.* 2011, Volkoff *et al.* 2005).

CCK is known to function as a satiety factor to suppress feed intake and stimulate termination of a meal (Gelineau and Boujard 2001, Himick and Peter 1994, Peyon *et al.* 1999, Volkoff *et al.* 2005). CCK was initially proposed to function as a satiety signal since injections of CCK result in reduced feed intake in rats (Gibbs *et al.* 1973). In addition, administration of the receptor CCK_A antagonist increased feed intake in rats (Corwin *et al.* 1991, Miesner *et al.* 1992, Reidelberger and Orourke 1989), mice (Weatherford *et al.* 1992) and rhesus monkeys (Moran *et al.* 1993). In fish, CCK has been identified in the central nervous system and peripheral tissues,

for example in goldfish, rainbow trout, spotted river puffer, *Tetraodon nigroviridis*, Japanese flounder, *Paralichthys olivaceus*, Atlantic herring, *Clupea harengus*, and Atlantic salmon (Jensen *et al.* 2001, Kurokawa *et al.* 2003, Murashita *et al.* 2009, Peyon *et al.* 1998, Peyon *et al.* 1999, Rønnestad *et al.* 2007). Increased concentration of brain *cck* (encoding gene of CCK; i.e. mRNA) after feed-intake has also been reported in goldfish (Himick and Peter 1994, Peyon *et al.* 1999) and Atlantic salmon (Rønnestad *et al.* 2010, Valen *et al.* 2011).

Table 1. List of peptides involved in the regulation of feeding behavior (Adapted from Ueta *et al.* 2003). Based on the knowledge from mammals this list is far from complete.

Anorexigenic peptides	Orexigenic peptides
Adrenomedullin	Neuropeptide Y (NPY)
Bombesin	Agouti-related peptide (AgRP)
Cholecystokinin (CCK)	Galanin
Calcitonin	Galanin-like peptide (GALP)
Cocaine-and amphetamine regulated transcript (CART)	Growth hormone-releasing hormone (GHRH)
Corticotropin releasing hormone (CRH)	Growth hormone (GH)
Ciliary neurotropic factor (CNTF)	Ghrelin
Gastrin-releasing peptide (GRP)	Melanin-concentrating hormone (MCH)
Galanin-like peptide (GALP)	Opioids
Glucagon-like peptide-1 (GLP-1)	Orexin-A/B
Glucagon	Prolactin
Melanocortin	Peptide YY (PYY)
Melanocyte-stimulating hormone (MSH)	
Neuromedin U	
Neurotensin	
Oxytocin	
Pituitary adenylate cyclaseactivating polypeptide (PACAP)	
Pro-opiomelanocortin (POMC)	
Somatostatin	
Thyrotropin-releasing hormone (TRH)	
Urocortin	
Vasoactive intestinal polypeptide (VIP)	

The understanding of the functions of neuropeptides involved in appetite and feeding behavior in cobia is yet missing. No information is available on gene expression of *npy* and *cck* and the role of NPY and CCK in regulation of appetite and feed intake in cobia. The understanding of the metabolism and utilization of lysine and arginine in cobia is still limited. There are no studies on to what extent dietary lysine to arginine ratio is linked to appetite regulatory neuropeptides and feed ingestion in cobia. Until now, no commercial diet specific for the species is available. In Vietnam, farmers are combining commercial diets for Asian seabass and grouper with trash-fish for feeding cobia. This aquaculture practice leads to poor feed conversion ratio, nutrient loss into the water, and more importantly it causes negative consequences on the environment.

2. AIMS OF THE STUDY

This study was done to assess impacts of different lysine to arginine ratios in diets with high inclusion levels of plant protein sources on appetite, feed intake, growth performance, protein and lipid deposition, and expression of brain *npy* and *cck* in juvenile cobia. The main aims were as follows:

I. Evaluate the palatability and feed intake of a diet with high inclusion levels of plant protein sources compared to commercial diets for cobia and assess the transition of ingested feed in the gastrointestinal tract 24 h post-feeding.

II. Evaluate whether a diet containing high plant protein inclusion supports growth at commercial rates, and if this diet is suitable to assess the effect of different ratios of lysine to arginine in juvenile cobia.

III. Determine to what extent different dietary ratios of lysine to arginine affects growth performance, lipid deposition and/or protein accretion in juvenile cobia.

IV. Determine if NPY and CCK in the brain are involved in the regulation of appetite changes during a meal in cobia.

V. Determine if different dietary ratios of lysine to arginine affect feed intake and regulatory pathways for appetite via brain expression of *npy* and *cck* in cobia.

VI. Determine if there is a time dependent adaptation to the diets in terms of feed intake and expression of brain *npy* and *cck*.

3. SUMMARY OF MATERIALS AND METHODS USED

3.1 Experimental diets

In the current study, one pilot experiment (PE) and three feeding trials (named experiment I, II and III) were carried out. One pelleted diet containing 480 g protein and 160 g lipid kg^{-1} dry matter produced at the University of Nha Trang was used for feeding juvenile cobia during acclimatization period of 7 days. Two available commercial diets (CD1, 480 g protein and 74 g lipid kg^{-1} dry matter; and CD2, 550 g protein and 95 g lipid kg^{-1} dry matter) used in commercial production of cobia in Vietnam and that provide good performance on production of juvenile cobia (Hung Van Lai, unpublished data, 2010) were selected. Three plant-based protein test diets (730 g plant ingredients; 108 g fishmeal kg^{-1}) with different lysine to arginine ratios were produced and extruded by EWOS Innovation AS, Norway. Appropriate amounts of crystalline lysine and arginine were added in the test diets to adjust the ratios of lysine to arginine. The ratio of lysine to arginine in one of the test diets was approximately 1.0 (1.1) and was termed BL/A diet (balanced lysine to arginine diet). While, the two other test diets were made imbalanced in the lysine to arginine ratios, including an increased dietary lysine to arginine ratio diet (named HL/A diet) and a reduced lysine to arginine ratio (LL/A diet). In the HL/A diet, the amount of lysine was 1.5 times (52.3/34.0) higher than that in the BL/A diet. In the LL/A diet, the amount of arginine was 1.4 times (43.1/31.0) higher than in the BL/A diet. One of the two amino acids (i.e. arginine in the HL/A diet, and lysine in the LL/A diet) were kept above predicted requirement and at the same level as in the BL/A diet. Therefore, the ratio of lysine to arginine in the HL/A- and LL/A diet was 1.8 and 0.8, respectively.

Further, the marine ingredients and fish oil in the LL/A- and HL/A diet were adjusted to assure that the three test diets contained the same inclusion level of plant ingredients, dietary protein, lipid, gross energy as well as a balanced amino acid profile above anticipated requirement (NRC 2011). The pellet size was 1.6-2.2 mm.

3.2 Experimental fish and water-circulation tanks

Juvenile cobia (3-7 g body weight, BW) collected from Hoang Ky rearing farm were transferred to indoor cylindrical fiberglass tanks (1 m^3) in a hatchery at Duong De, Nha Trang, Vietnam with the density of 200 individuals/m^3. Four different batches of juvenile cobia were used in the four sequential experiments in this study. The fish were fed *ad lib* at 8:00 and 17:00 by a diet (480 g protein and 160 g lipid kg^{-1} dry matter) produced at the University of Nha Trang during the acclimatization period of 7 days. After the acclimatization period, cobia were sorted out and

fish of similar BW (8.0±0.1 g) were used for the pilot experiment and for the other three experiments (9.3±0.1 g, BW for experiment I; 8.4±0.1 g, BW for experiment II; and 10.5±0.1 g, BW for experiment III). The fish were randomly distributed into the experiment tanks and kept for 48 h (in the pilot study) and 4 weeks (experiment I) or for 6 weeks (experiments II and III).

The experimental tanks used were rectangular fiberglass tanks (0.4x0.5x0.6 m), with 110 L water filled, setting under a water recirculation system with continuous aeration. Each of the diets in experiment I (Paper I), II (Paper II) and III (Paper III) were randomly assigned to three tanks. Input water from a filtered fiberglass tank (1.0x1.0x2.0 m) went through plastic pipes to rearing tanks (0.2 L second^{-1}). Output water from the rearing tanks was collected by perpendicular pipes (Ø 27 mm) in the middle of each tank. Output water was then filtrated in a fiberglass tank (1.0x1.5x 2.0 m), before it were pumped back in to the filtered fiberglass tank (for input water). Seawater was pumped into a reservoir (24 m^3), and was desedimented and chloride treated before coming into the recirculation system. Water in the recirculation system was renewed every 2-3 days depended on environmental parameter analyses. In experiment I, water temperature was 30.5±2.3 °C (mean±SD), salinity was at 30±3.1 g L^{-1}, pH at 7.8-8.3, oxygen at 3.8±0.5 mg L^{-1} and $NH_3 \leq 0.1$ mg L^{-1}. While, these parameters for water in experiments II and III were 29.2±2.8 °C, salinity was 28±3.1 g L^{-1}, pH 7.8-8.3, oxygen 4.6±0.5 mg L^{-1}, $NH_3 \leq 0.03$ mg L^{-1}. The experimental tanks were covered by a fishing-net on the top to prevent any cobia jumping out of the experimental system.

3.3 Feeding trials

Pilot experiment: One hundred and sixty two cobia were distributed in to twenty seven tanks (12 individuals/tank) and starved for 24 h. Cobia was randomly assigned to the three diets. Cobia were fed *ad lib* by hand at 8.00. Fifty four unfed cobia were also included as a reference (control group)

Experiment I: Based on results from the pilot experiment, the BL/A diet was chosen together with two commercial diets (CD1 and CD2) for experiment I to compare growth, and protein and lipid deposition. The same regime as used in the acclimation period was adopted for 4 weeks (Paper I).

Experiment II: In this next trial, BL/A diet and two other test diets LL/A- and HL/A (described above) were compared to the commercial diet, which showed best results in experiment I (CD2), to compare growth performance. This experiment lasted for 6 weeks (Paper II).

Experiment III: Cobia were fed the four diets that were used in experiment II for 6 weeks. Brain samples were collected at week one and week six to determine change in *npy* and *cck* of pre-feeding (unfed) and post-feeding (fed) cobia consuming different ratios of lysine to arginine in association with feed intake and appetite in short- and long-term (Paper III).

3.4 Sampling procedure

Prior to exposure to any sampling, juvenile cobia were anesthetized by MS-222 solution (0.4 g L^{-1}). Individual body weight and standard length were measured to the nearest 0.1 g and 0.1 cm, while visceral organ, liver and content in the gastrointestinal (GI) tract were measured to the nearest of 1 mg. Fig. 4 depicts juvenile cobia with different dissected parts.

In the pilot experiment, six fed cobia the CD1-, CD2- and BL/A diet, were dissected for collection of ingesta and chyme from the stomach, midgut and hindgut at 0, 0.25, 0.5, 1, 2, 4, 8, 16 and 24 h postprandial. Six unfed cobia from the control group were also dissected for collection of chyme in the GI-tract at the same postprandial time. Therefore, control fish had fasted for 48 h at the time of the final sampling. The fish's GI-tract was dissected and carefully separated in stomach, midgut and hindgut to avoid loss of content. Chyme and ingesta in these segments were carefully collected and transferred onto pre-weight aluminum foils. The collected contents in the GI-tract were dried at 105 °C in the oven (Clayson Laboratory Apparatus Pty. Ltd.) for 24 h for determining dry weight basic.

At the end of experiment I, cobia was starved for 24 h before measuring body weight, standard length and relative weight of visceral organs (for calculation of viscerosomatic index, VSI and hepatosomatic index, HSI). Efferent branchial artery blood was collected 24-h postprandial. Whole body, lateral muscle (standardized between pectoral fin and anal fin) and liver were collected and stored in freezer (-80±3 °C) until analyzed. Similar protocol of sampling was used in experiment II, except blood samples was collected at 5 h postprandial. Uneaten feed was recorded for calculation of feed conversion ratio, feed intake, and protein productive value (for details, see Papers I, and II).

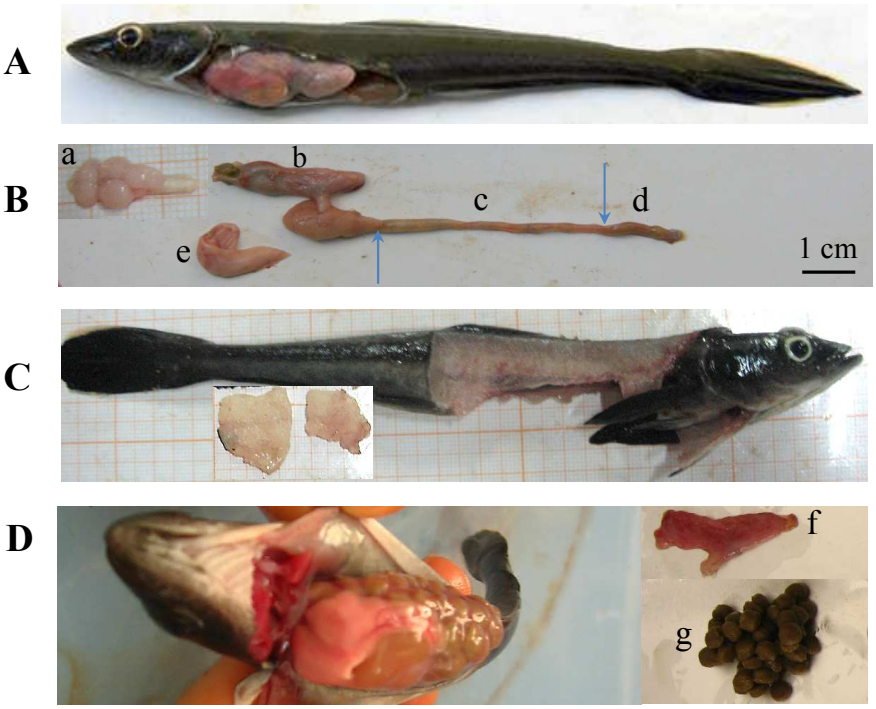

Fig. 4. Schematic diagram showing the dissection of cobia for collecting samples. **A**, the juvenile cobia with body cavity opened; **B**, the brain and viscera; a, is the whole brain; b, stomach; c, midgut; d, hind gut; e, liver. Arrows indicate two cutting sites to separate the midgut and hindgut from the GI-tract. During dissection, the gut was carefully stretched out, then the hindgut was identified from the GI terminus to the first folded-gut site, and the midgut was identified between the hindgut and the outlet of the stomach (pylorus); **C**, the cobia with two pieces of lateral-muscle sampled from each side of the body; **D**, the cobia with a filled stomach immediately (0 h) after feeding to satiation; f, empty stomach; g, the ingested pellets.

In experiment III, cobia were sampled at week one and week six. Both body weight and standard length were recorded. Cobia's whole brain was collected, and emerged in RNA later solution for RNA extraction. Content in the stomach was also collected for stomach filling calculation (for details, see Paper III).

3.4 Sample analysis

Proximate analysis (Paper I and II)

Moisture was determined by drying out samples at 105 °C for 24-36 h until constant weight in a moisture extraction oven (Clayson Laboratory Apparatus Pty. Ltd.) then kept in NUNC boxes in the freezer until analyzed. Nitrogen was analyzed by Leco FP-528 instrument and crude protein was calculated assuming amino acids contain 16% nitrogen (Espe *et al* 2006). In short 0.1-0.2 g homogenized samples was weight into a foil that placed on a cup holder. The foil with sample was twisted to seal and then put in to the sample loading head of Leco FP-528 instrument where it was burned to nitrogen gas. Total lipid of the diets and biological samples was analyzed gravimetrically after extraction with ethylacetate as described (Espe *et al*. 2006). In short, 5.0 g of the samples was added 30 mL ethylacetate – isopropanol solution (30% isopropanol) and shaken for 2 h (KIKA® WERKE- HS501). The sample was filtered and an aliquot of 5 mL was evaporated at 70 ^{o}C for 2 h in the oven (Termaks).

Amino acid analysis (Papers I and II)

Amino acid composition in feed was analyzed by UPLC (Ultra Performance Liquid Chromatography) after being hydrolyzed for 22 h at 110 °C. Aliquot of 500 µL sample was centrifuged at 60 °C for 120 minutes in the Centrivap Concentration before added 5 mL distilled water, shaken thoroughly and then 1.0 mL sample was transferred to 1.5 mL tube through the filter. An aliquot of 10 µL sample solution was transferred into 12 x 32 mm glass screw neck vial (Quick Threat) that pre-filled with 70 µL Borat buffer (AccQ.Tag ^{TM} Ultra Derivatization Kit). The sample vial was added with 10 µL external standard (500 pm/mL of hydroxylproline, taurine -Sigma- and amino acid standard H –Pierce) following by adding 20 µL reaction solution (AccQ.Tag ^{TM} Utra Derivatization Kit-), vortexed for 10 seconds and heated at 56 °C for 10 minutes. The samples were put in vial holding tray and loaded in to the sample manager of UPLC system. The concentration of amino acids was quantified by TUV (Tunable Ultraviolet) detector and Empower Build 2154 software. While amino acid composition in deproteinized plasma was determined on an Biochrom 20 plus Amino Acid Analyzer (Amersham Pharmacia Biotech, Sweden) equipped with a lithium using post column derivatisation with ninhydrin as described (Espe *et al*. 2006). Norleucine was added as an internal standard. The amino acids were quantified using a standard containing the amino acids of interest as well as urea and ammonia (Sigma Aldrich, Germany).

Cloning and RT-qPCR (Paper III)

Brain samples were homogenized in TRI Reagent using Lysing Matrix D tubes (MP Biomedicals, Solon, OH, USA) in a FastPrep FP120 (Savant Instruments, Holbrook, NY, USA), and then total RNA was isolated using TRI Reagent according to the manufacture's protocol (Sigma, USA). Concentration and purity of extracted RNA were measured using a NanoDrop ND-1000 (Thermo Scientific, USA). Quality of RNA was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA) in conjunction with Agilent RNA 6000 Nano kit (Agilent Technologies). Extracted RNA from all samples were determined the RNA integrity number (RIN) following the Agilent RNA 6000 Nano Assay protocol (Agilent Technologies). Extracted RNA samples were then used for cloning of *npy* and *cck* and for Real time quantitative PCR (Q-PCR) using β-actin as the reference gene. Q-PCR was performed using a CFX 96™ Real Time System (Bio-Rad), the thermal profile was 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 sec, 58 °C for 30 min, 72 °C for 30 sec, with a final denaturation step of 10 sec at 95 °C. A melting curve was generated for each run, to verify the absence of primer dimers and the specificity of the PCR reaction (65 – 95 °C with an increment of 0.5 °C and holding for 5 sec). SYBR was used as a reporter (iQ™ SYBR® Green supermix; Bio-Rad) (for details, see Paper III).

3.5 Statistical analysis

Data was analyzed by the statistical program SPSS for Windows (IBM® SPSS® Statistics version 19). Values are given as tank means ± SEM (standard error of the mean). ANOVA was used to test any differences between dietary treatments using tanks as the statistical unit. If differences were obtained ($p<0.05$), the Tukey's test was used to evaluate the differences between treatments. Prior to applying ANOVA, a Levene's test was done for testing the homogeneity of variances of the dependent variables. When the data was found inhomogeneous in variance, a Welch's test was used to determine any differences between dietary treatments.

For the mean normalized expression (MNE) data, a natural logarithm (Ln) transformation was performed as MNE values were inhomogeneous. Ln(MNE) values were homogenous in variance and normal distributed. A two way ANOVA was used to test for differences in gene expression before and after feeding within and between the dietary treatments.

4. MAJOR FINDINGS

4.1. Voluntary feed intake and transition of ingesta in gastrointestinal tract

In the PE, juvenile cobia showed high appetite when they were offered the two commercial diets, and the plant-based protein test diet with balanced lysine to arginine ratio (BL/A). Analysis of the contents from the stomach indicated that juvenile cobia had a feeding rate of 5.3±0.3% BW for CD1-, and BL/A diet, and slightly higher for the CD2 diet (5.4±0.4% BW). No significant differences in stomach filling occurred between cobia fed the BL/A diet and the two commercial diets. Dry matter in the stomach of unfed cobia was stable as a minimum level (1.88-2.83 mg or 0.03-0.04% BW) within the time of the experiment. Significantly higher stomach filling in fed cobia compared to unfed cobia indicated the good palatability of the plant-based protein diet and both the commercial diets.

Gastric evacuation rates in juvenile cobia fed three diets in Fig. 5 could be fitted by the exponential function $Y_T=V_0\, e^{-b(x)}$ (V_T, volume of feed at time T; V_0, volume of feed at time 0; b, the instantaneous evacuation rate; and x, time postprandial; $R^2>0.95$). One hour after a single meal, most of the ingesta was still in the stomach (89; 88 and 91% estimated from dry matter basic for CD1-, CD2- and BL/A diet, respectively), with only a small fraction transferred to the midgut (MG) and hindgut (HG). Stomach was gradually emptying, and 36-41 % of ingested feed was transferred to the further parts of the GI-tract at the 4 h after a meal. Between 77 to 80% of stomach contents was evacuated to the lower parts of the GI-tract at 8 h, and most of consumed feed (98%) was emptied out of the stomach around the 16 h postprandial (Fig. 5). Based on gastric evacuation results at 8 h postprandial, it could be inferred that the return of appetite in cobia was within this period after being fed to satiation.

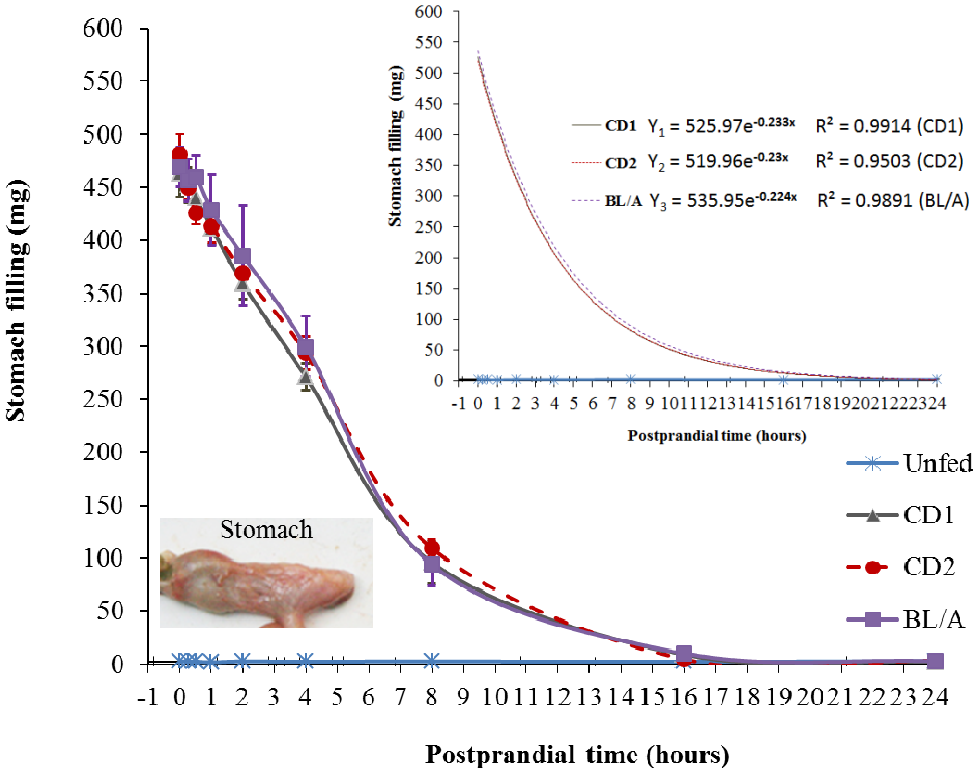

Fig. 5. Stomach filling (dry mass) in juvenile cobia fed different diets postprandial. Data are presented as means (n=6) at selected time points after feeding. Sampling started from time 0/ just after cobia fed to statiety. Vertical bar indicates ±SEM. The upper graph (insert) shows calculated gastric evacuation based on exponential fit for each diet. The equation for the relationship between stomach content (Y) over time (x) postpradial in cobia fed the CD1 diet was $Y = 526e^{-0.233x}$ ($R^2 = 0.9914$); CD2 diet, $Y = 520e^{-0.23x}$ ($R^2 = 0.9503$); and BL/A diet, $Y = 536e^{-0.234x}$ ($R^2 = 0.9891$).

Dry contents of chyme in the MG gradually increased and peaked at 4-6 h postprandial, and then gradually declined to the level close to the unfed cobia at 16 h postprandial (Fig. 6).

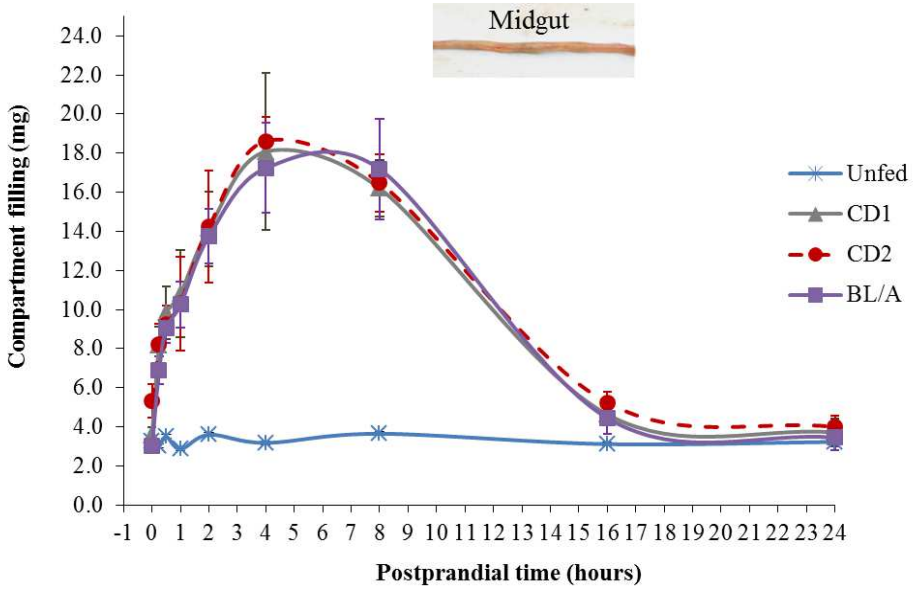

Fig. 6. Chyme content (dry mass) in midgut of juvenile cobia fed different diets postprandial. Data are presented as means (n=6) at selected time points after feeding. Sampling started from time 0/ just after cobia fed to statiety. Vertical bar indicates ±SEM.

At 0.5 h postprandial, content of the chyme in the HG rapidly increased to the highest level observed during the study, and stabilized at this level within the 4-16 h, followed by a rapid decrease to the minimum level similarly to unfed cobia around the 24 h postprandial (Fig. 7).

It should be noted that there was a methodological challenge regarding sampling the complete contents of the GI-tract. The pyloric caeca is a complex compartment, and despite the relatively large appearance (Fig. 4) the intraluminal volume of each caecum was very small and impossible to empty. The chyme stored in the caeca appeared to be relatively small when stripping was tested, but these trials resulted in crushed tissue and unreliable and mixed matter (tissue and chyme). Also, the remaining content from GI-tract in 24-h and 48- h starved cobia shows that there was still some leftover chyme (unfed, Figs 5, 6, 7). The composition of this is not known, but might probably be indigestible matter with some bile due to the yellow color.

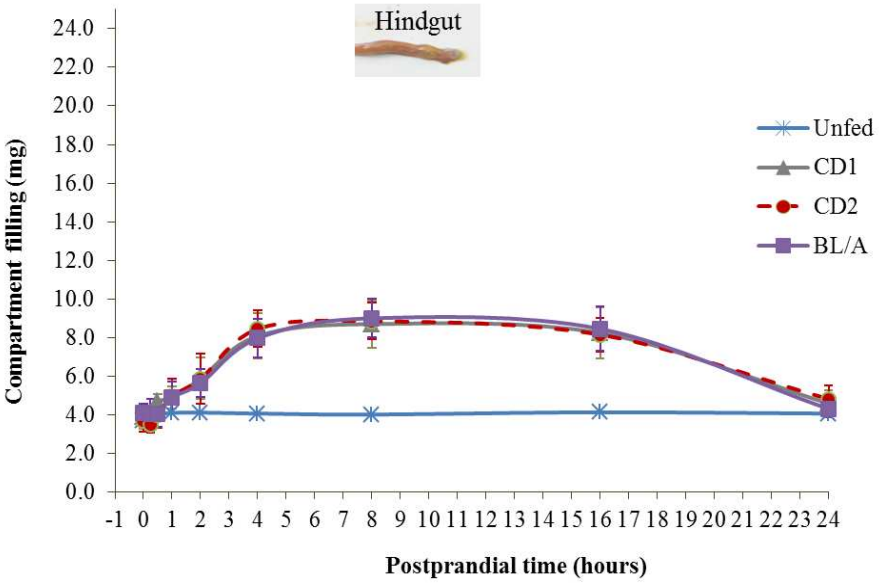

Fig. 7. Chyme content (dry mass) in hindgut of juvenile cobia fed different diets postprandial. Data are presented as means (n=6) at selected time points after feeding. Sampling started from time 0/ just after cobia fed to statiety. Vertical bar indicates ±SEM.

4.2 Plant-based protein diet, and growth, and deposition of protein and lipid

Based on results from the pilot experiment, the CD1-, CD2- and BL/A diet were included in the next trial (experiment I). Juvenile cobia grew well when they were offered the CD1- , CD2- and BL/A diet, and there were more than tripled their initial body weight during four-weeks (Paper I). Cobia fed the BL/A diet showed weigh gain, protein gain, and feed conversion ratio comparable to cobia fed the CD2 diet (Paper I). Cobia fed the BL/A diet obtained better body weight, protein gain and feed conversion ratio than cobia fed the CD1 diet. Higher body lipid deposition was observed in cobia fed both of the commercial diets as compared to cobia fed the BL/A diet. No differences in protein deposition or condition factor (CF), viscerosomatic index (VSI) or hepatosomatic index (HSI) were observed between cobia fed any of the three diets.

4.3 Lysine to arginine ratios, and plasma free amino acids

Total plasma free amino acids (FAAs) concentration 24 h postprandial did not differ among cobia fed the CD1, CD2 and BL/A diet (Paper I). Plasma indispensable FAAs consisted mainly of threonine, lysine, valine and leucine. Plasma dispensable FAAs comprised dominantly glycine and alanine.

In experiment II, total concentration of plasma FAA profiles of 5 h postprandial varied among cobia fed the CD2-, BL/A-, HL/A- and LL/A diet, but did not reflect the dietary ratios of these two amino acids (Paper II).

4.4 Lysine to arginine ratios, and growth, and deposition of protein and lipid

According to results from the experiment I, the BL/A diet together with two other plant-based protein diets, LL/A- and HL/A diets, were used in experiment II for determining impacts of different lysine to arginine ratios on growth performance and nutrient accretion. The commercial diet, CD2, which showed better results in cobia (paper I), was also included as a reference for growth in experiment II (Paper II). The diets were fed during a period of six weeks. Cobia fed the BL/A diet showed growth comparable to those fed the CD2 diet that was in line with results from experiment I. There were no significant differences in final body weight, weight gain, feed conversion ratio or protein gain between cobia fed the BL/A diet and the CD2 diet. However, growth of cobia fed the BL/A diet was better than fish fed both the imbalanced diets (HL/A- and LL/A, Paper II).

In addition, protein gain of cobia fed the BL/A diet was significantly higher than in cobia fed both the HL/A- and LL/A diet (Paper II). No significant differences in protein efficiency ratio (PER) were observed between cobia fed the CD2, BL/A- and HL/A diet. While, cobia fed the LL/A diet showed lower PER compared to cobia fed any of other three diets. Cobia fed the LL/A diet also had lower muscle protein and protein productive value (PPV) compared to cobia fed the commercial control diet, while cobia fed the BL/A- and HL/A diet showed intermediary in PPV. Further, cobia fed the HL/A- and LL/A diet showed higher HSI than those fed the BL/A diet. No significant differences in CF or VSI were observed between cobia fed any of the different lysine to arginine ratio diets.

4.5 Lysine to arginine ratios, and appetite and feed intake

Lysine to arginine ratios influenced the voluntary feed intake (FI) in juvenile cobia. In experiment II, daily FI in cobia fed the BL/A diet was comparable to those fed the CD2 diet, but significantly higher than those fed the HL/A- and LL/A diet (Paper II). Further, cobia fed the LL/A diet showed poorer feed conversion ratio (FCR) than cobia fed the BL/A diet or the HL/A diet.

In experiment III, cobia fed the HL/A diet showed lower daily FI compared to cobia fed the control diet (CD2 diet), while daily FI in cobia fed the LL/A diet was lower than that in cobia

fed both the BL/A and the CD2 diet (Paper III). Among the diets, cobia had less stomach filling at week one due to smaller size compared that in cobia at week six (Fig. 8). At week one, cobia fed the LL/A diet showed less stomach filling compared to cobia fed the commercial control diet (i.e. CD2). No significant differences in stomach filling was observed when cobia were offered the BL/A-, LL/A- and HL/A diet at week one and at week six.

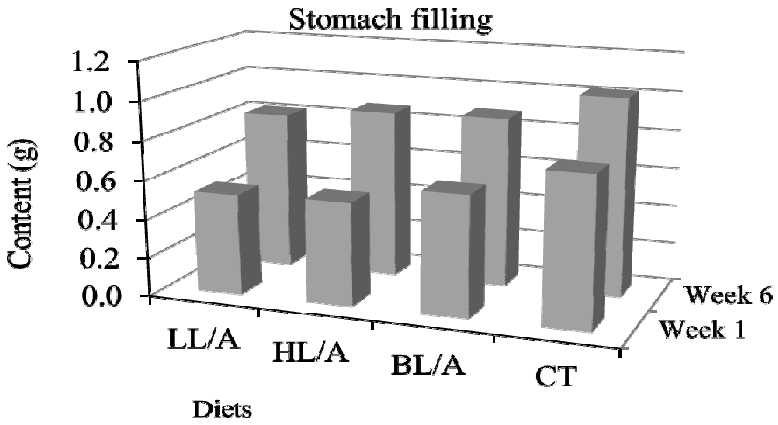

Fig. 8. Stomach content (dry mass) in juvenile cobia fed different diets at week 1 and week 6. Due to smaller size, cobia at week one had less stomach content compared to that in cobia at week six for all four diets (*P<0.05; Two-way ANOVA*). At week one, cobia fed the LL/A diet had less stomach content than cobia fed the CT diet 0 h after feeding, while cobia fed the BL/A- and HL/A diet had intermediate stomach content. No significant differences in stomach filling occurred between cobia fed the four diets at week six.

4.6 Feeding status and dietary lysine to arginine ratios, and brain npy *and* cck *expression*

Feeding affected the expression of *npy* in cobia. At week one, levels of brain *npy* in unfed cobia were significantly higher than in fed cobia with the BL/A-, HL/A- and CD2 diet, while no significant differences in brain *npy* expression were detected between unfed and fed cobia the LL/A diet (Paper III). At week six, brain *npy* levels in unfed cobia were significant higher than fed cobia with all diets. Comparison gene expression levels from cobia's brain between different dietary treatments showed that *npy* expression did not differ significantly among unfed cobia or among fed cobia at short-term and long-term when the diets were introduced (Fig. 9.1 and 9.2).

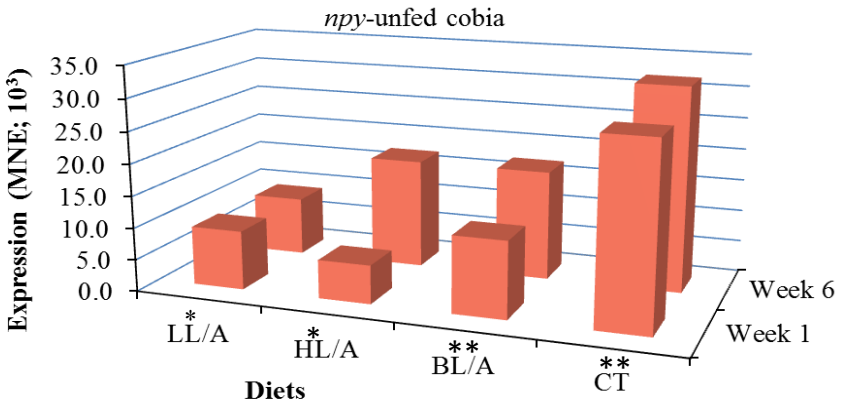

Fig. 9.1. Mean normalized expression (MNE) of brain *npy* in unfed (pre-feeding) cobia at week 1 and week 6. Differences in MNE between dietary treatments were not significant (*P>0.05; Two-way ANOVA*). Asterisk (*) indicates diet gave significant differences in MNE between pre-feeding and fed cobia at week six, while double asterisk (**) indicates diet gave significant differences in MNE between pre-feeding and fed cobia at both week one and week six.

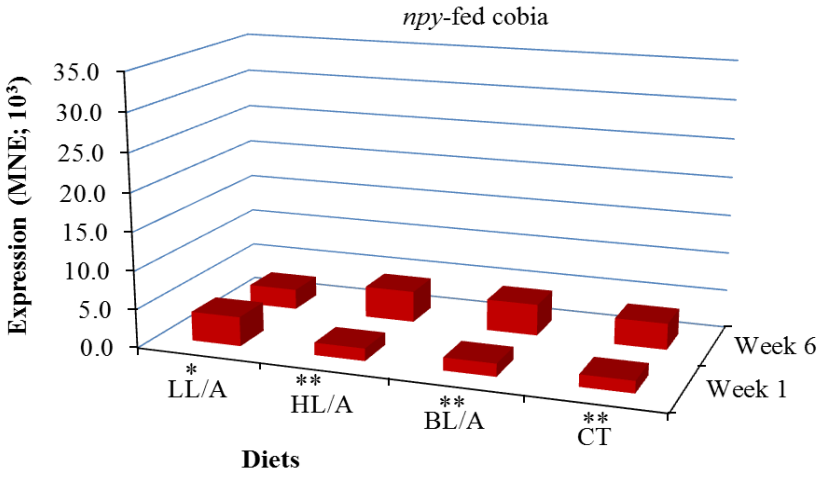

Fig. 9.2. Mean normalized expression (MNE) of brain *npy* in fed cobia (15 min after fed to satiation) at week 1 and week 6. Differences in MNE values between dietary treatments were not significant (*P>0.05; Two-way ANOVA*). Asterisk (*) indicates diet gave significant differences in MNE between pre-feeding and fed cobia at week six, while double asterisk (**) indicates diet gave significant differences in MNE between pre-feeding and fed cobia at both week one and week six.

No significant differences in brain *cck* expression were observed between unfed cobia and fed cobia at short-term and long-term (Fig. 10.1 and 10.2) when the diets were introduced. Comparison gene expression levels from cobia's brain between different dietary treatments showed that *cck* levels were not significant different among unfed cobia or among fed cobia at short-term and long-term when the diets were introduced.

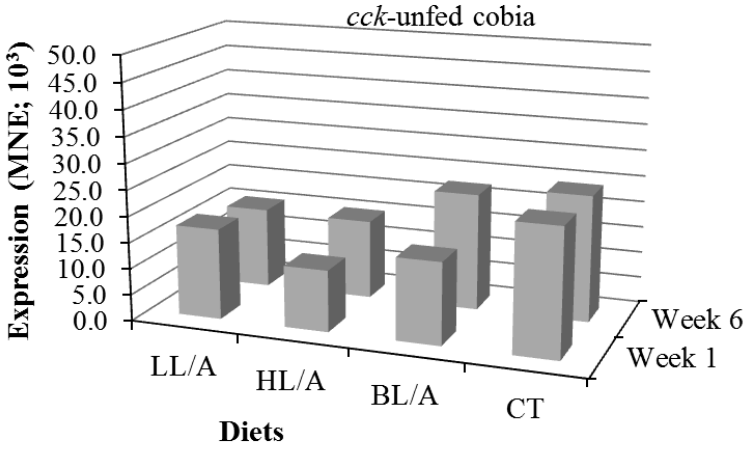

Fig. 10.1. Mean normalized expression (MNE) of brain *cck* in unfed cobia (pre-feeding) at week 1 and week 6. Differences in MNE values between dietary treatments were not significant (*P>0.05; Two-way ANOVA*).

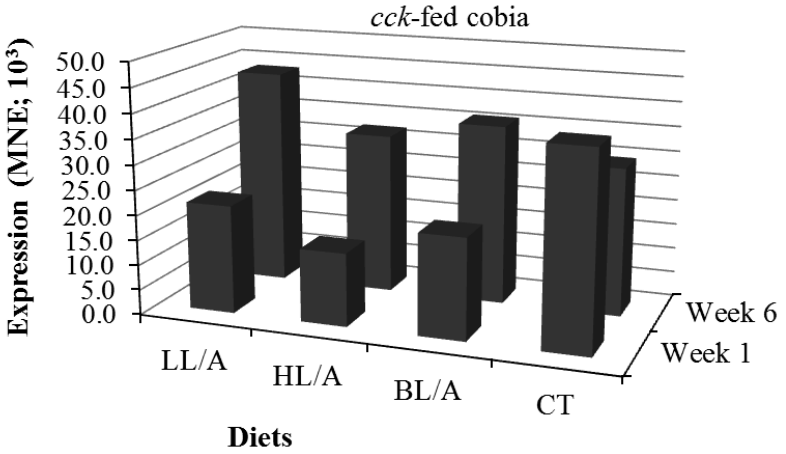

Fig. 10.2. Mean normalized expression (MNE) of brain *cck* in fed cobia (15 min after fed to satiation) at week 1 and week 6. Differences in MNE values between dietary treatments were not significant (*P>0.05; Two-way ANOVA*).

5. GENERAL DISCUSSION

5.1 Voluntary feed intake and transition of ingesta in gastrointestinal tract

In aquaculture, understanding the rate of digestion in association with gastric evacuation rate may help to predict the return of appetite (Riche *et al.* 2004), and figure out appropriate feeding strategies for better feed intake and feed efficiency by administering food as soon as appetite has returned (Grove *et al.* 1978, Lee *et al.* 2000). Several mathematical models have been proposed to estimate gastric evacuation rate, for example linear model (Bromley 1988, Tyler 1970), exponential model (He and Wurtsbaugh 1993, Riche *et al.* 2004, Stubbs 1977), square root or linear model (Jobling 1987, Lambert 1985, Pandian 1967). Though, there is still controversial as to which model would be the most appropriate applicable one due to the variation of factors affecting gastric evacuation rate. For example, Jobling proposed that small particles of a low energy density, e.g. zooplankton, were exponentially evacuated, while large particles of high energy density, e.g. fish prey, were linearly evacuated (Jobling 1987). Plotting the gastric evacuation curves for the data obtained in the pilot experiment indicates an exponential relationship between the stomach content and the time postprandial in cobia (Fig. 5). These findings were in line with the model proposed that cobia show gastric evacuation rate in an exponential function (He and Wurtsbaugh 1993, Riche *et al.* 2004, Stubbs 1977). However, feed makers were not available in the present study and cobia were fed pellets to satiety only one meal, thus the precision of the estimated model is limited. Further studies using inert indicators such as titanium dioxide (TiO_2) or ferric oxide (Fe_2O_3) (Riche *et al.* 2004, Richter *et al.* 2003) in combination with different diet composition and feeding regimes are required to accurately estimate the gastrointestinal transit kinetics in cobia.

The pilot experiment was not designed to test such models in estimating gastric evacuation and feed consumption in cobia. Rather, this experiment aimed to evaluate whether the CD1-, CD2-, and BL/A diet would be well accepted by cobia, thus if they could be used for growth studies. In the pilot experiment, cobia had consumed 5.3-5.4% body weight (BW) when they were first offered the CD1-, CD2- and BL/A diet. This indicated good palatability of all three diets when compared to the recommendations made by Sun and coworkers that feeding rate should be from 9% BW day^{-1} in cobia 10–20 g (Sun *et al.* 2006); and reduced to 2–3% BW day^{-1} in cobia of 100–200 g BW for better growth and feed efficiency (Liao *et al.* 2004).

The evacuation time of the ingesta through the GI-tract is in association with the absorption of nutrients following feeding (Dabrowski 1983, Fletcher 1984, Talbot *et al.* 1984). Generally,

cold water fish require longer time to achieve complete digestion than warm water fish species, consequently warm water fish show shorter evacuation time of ingesta though the GI-tract compared to cold water fish (Smith 1989). Atlantic salmon showed gut transit time of 60 h (Talbot *et al.* 1984), while this in hybrids sarotherodon, *Oreochromiss niloticus x Sarotherodon areus* was 24 h (Ross and Jauncey 1981). Time required for gastric evacuation in common dab, *Limanda limanda*, and black rockfish, *Sebastes melanops* was 15 h and 76 h, respectively (Brodeur 1984, Fletcher 1984). In the present experiment, about 80% of the stomach content had been evacuated to the lower part of the GI-tract at the 8 h postprandial. The return of appetite is closely related to the GI emptying (Huebner and Langton 1982, Sims *et al.* 1996, Vahl 1979). Hunger in satiety feeding fish recovers when 80-90% of the stomach content has been evacuated (Grove *et al.* 1978, Riche *et al.* 2004, Valen *et al.* 2011), as orexigenic signals in the GI-tract may increase when most of the content in the stomach evacuates, while anorexigenic signals decrease accordingly (Valen *et al.* 2011). Results from the pilot experiment, together with literature data suggested that the appetite in cobia have returned 8 h after satiety feeding. This was supported by the fact that cobia had good appetite in the 2nd feeding of the day.

In summary, results from the pilot experiment suggested that juvenile cobia showed high appetite to the CD1-, CD2- and BL/A diet, providing possibility of use these diets for growth study. Time required for the return of appetite in cobia was within 8 h after feeding to satiation.

5.2 Plant-based protein diet, and growth, and deposition of protein and lipid

Fishmeal offers several advantages, e.g. high protein levels, well balance of amino acid profiles, particularly high level of indispensable amino acids compare to other protein sources. Thus, traditionally formulated diets include fishmeal as the main source of protein (Venero *et al.* 2008). Commercial diets for marine fish, particularly for larvae and other early stages of the life cycle, often contain high level of fishmeal inclusion (Miles and Chapman 2006), with the aim to enhance growth and survival rate. In the present study, the formulations of the two commercial diets (i.e. CD1 and CD2) are unknown. However, as taurine and OH-proline are abundant in animal tissues (Espe *et al.* 2012b, Park *et al.* 2002), the higher level of fishmeal is included in diets, the higher the concentration of these two amino acids will be. Concentration of the two amino acids reduces as fishmeal is replaced with plant protein ingredients (Espe *et al.* 2012b). Considerably higher concentration of taurine and OH-proline in both the CD1- and CD2 diet (Table 2; Paper I) indicates a higher inclusion level of animal protein sources in the two commercial diets, compared to the plant-based protein test diets.

In the current study, cobia fed the BL/A diet that contained high plant protein inclusion (730 g plant ingredients, 118 g fishmeal kg^{-1} diet) showed growth comparable to cobia fed the CD2 diet, but better than cobia fed the CD1 diet (Table 4; Paper I). Concentration of protein and lipid in the CD2 diet and BL/A diet were higher than in the CD1 diet, which may have resulted in better growth performance in cobia fed the CD2 diet and BL/A diet, compared to cobia fed the other commercial diet, CD1. Several studies reported different fish species respond differently to the inclusion of alternative protein sources in the diets. Generally speaking, omnivorous fish species tolerate a higher level of inclusion of plant protein sources in the diets, compared to carnivorous species that have been embedded in feeding animal protein sources in the wild throughout the life history. Because plant protein is not well balanced in indispensable amino acids as compared to fishmeal (Venero *et al.* 2008), one often uses a blend of plant protein ingredients in combination with supplementation of crystalline amino acids when formulating the diets. By doing so, the requirements and/or mimic the amino acid profiles of the fishmeal based diets could be met, and maximum growth and feed utilization would be achieved in fish fed plant-based protein diets. A combination of corn gluten meal, soy protein concentrate and wheat gluten could totally successfully replace fishmeal in the diet for gilthead seabream (Kissil and Lupatsch 2004). Up to 660 g kg^{-1} of fishmeal could be replaced by blends of vegetable proteins without negative effects on growth performance of rainbow trout (Gomes *et al.* 1995). Rainbow trout showed good growth and nutrient utilization when they were offered diets containing soybean protein concentrate with methionine supplementation, compared to fishmeal-based diet (Kaushik *et al.* 1995). These results were supported by Rodehutscord *et al.* (1995) on this species that plant-based protein diet added crystalline amino acids to balance amino acids towards the requirement did not negatively affect growth and nutrient utilization. Red drum, *Sciaenops ocellatus* performed well on a diet of 900 g kg^{-1} soybean meal, compared to those fish fed fishmeal-based diets (McGoogan and Gatlin 1997). A total replacement of fishmeal by soybean meal, supplemented with methionine and fortified with minerals did not cause negative effects on growth and feed utilization in rohu (Khan *et al.* 2003). While, Atlantic salmon fed a plant based diet containing 50 g kg^{-1} fishmeal performed equally well as fish fed the fishmeal-based control diet (Espe *et al.* 2007). A replacement of 300 g kg^{-1} soybean meal or 360 g kg^{-1} full-fat soybean in the diets for Atlantic halibut did not cause negatively effects on growth rate, feed intake, feed efficiency protein retention and energy retention, as well as intestinal morphology and pathological reactions in the species (Grisdale-Helland *et al.* 2002, Murray *et al.* 2010).

In juvenile cobia, studies have shown that a replacement of up to 400 g kg^{-1} fishmeal protein with soybean meal protein would not affect weight gain and feed efficiencies (Chou *et al.* 2004). Cobia showed high growth rate and feed efficiency when they were fed on diet containing 335 g kg^{-1} fishmeal and 285 g kg^{-1} toasted defatted soybean meal; comparable to those fish fed the fishmeal-based control diet (Romarheim *et al.* 2008). Similar results were reported by Lunger and coworkers, though a blended diet containing 80 g kg^{-1} fishmeal diet reduced growth and feed efficiency in this species (Lunger *et al.* 2007b). In addition, juvenile cobia also showed a high capacity to utilize phosphorus present in the ingredients. It has been reported that apparent dry matter digestibility for juvenile cobia ranged between 0.60-0.88 for animal products and corn gluten meal, and between 0.59-0.71 for soybean meal, peanut meal, and rapeseed meal (Zhou *et al.* 2004). In the current study, cobia fed the plant-based protein diet with amino acids balanced towards the requirements and balanced in lysine to arginine ratio showed growth comparable to cobia fed the commercial CD2 diet, but better than cobia fed the commercial CD1 diet. These results indicate that cobia could growth and utilize plant ingredients well.

Poorer growth performance observed in cobia fed the CD1 diet compared to cobia fed the CD2- or BL/A diet may due to the fact that the CD1 diet had lower indispensable to dispensable amino acid ratio (IAA/DAA) than the CD2- and BL/A diet (Table 3, Paper I). Imbalanced amino acid diets have been reported to reduce growth performance rainbow trout (Yamamoto *et al.* 2000). Green and coworker reported that weight gain, feed efficient ratio and nitrogen retention in rainbow trout generally increased when the dietary ratio of indispensable to dispensable amino acids increased between 0.30 to 1.33, but growth performance decreased when the ratio increased above 1.94 (Green *et al.* 2002). Similar results are also reported in gilthead seabream (Gomez-Requeni *et al.* 2003). In our study, the BL/A diet had an IAA/DAA (0.80) comparable to commercial diet CD2 (0.77), while commercial diet CD1 had a lower ratio (0.74). This may contribute to the better growth performance obtained in cobia fed the high plant protein test diet and commercial diet CD2 as compared to fish fed commercial diet CD1. However, further studies are required to acquire knowledge of the impacts of dietary IAA/DAA ratio on cobia.

Cobia fed the BL/A diet in the current study did not differ from cobia fed the CD1- and CD2 in hepatosomatic index (HSI) and viscerosomatic index (VSI) or condition factor (Table 4; Paper I). These findings were in accordance with results from other studies on cobia fed diets with high inclusion levels of soybean protein (Zhou *et al*. 2005), and on Atlantic salmon fed plant-

based protein diets (Espe *et al*. 2006). In addition, in the current study, cobia fed the high plant protein inclusion test diet deposited less lipid in the whole body, compared to cobia fed the two commercial diets; and less or equal lipid deposition in liver. European seabass deposited more lipid when fed high plant protein inclusion diets (Kaushik *et al.* 2004). In contrary to this, Atlantic salmon fed plant-based protein diets deposited significantly less lipid than those fish fed fishmeal-based diet (Espe *et al.* 2006). In cobia muscle lipid are reported to increase as dietary soybean meal increased (Chou *et al.* 2004) or being unaffected (Zhou *et al.* 2005) . It has been reported that lipid accumulation in the liver and/or peritoneal cavity affects the health status of animals (Craig *et al.* 1999, Flood *et al.* 1996, Mathis *et al.* 2003, Tucker *et al.* 1997). However, whether there are such negative influences of lipid accumulation on health status of cobia requires further studies. Our findings support the hypothesis that there are differences between species in the amount of fishmeal that can be replaced, which partly are likely related to their natural life history and food choices in nature.

In the present study, cobia fed the high plant protein test diet (BL/A) did not differ in protein efficient ratio and protein productive value from that in the fish fed either the CD1- or CD2 diet, while cobia fed the BL/A diet showed better FCR and protein gain than the fish fed the CD1 diet (Table 4 and 5; Paper I). These findings suggested that the plant proteins from the test diet used were deposited sufficiently. According to Espe and coworkers, plant protein diets can be well utilized in fish providing the dietary amino acids are balanced towards the requirement (Espe *et al*. 2006; 2007). This implies that the amino acid profile has a more important role than crude dietary composition. The experiment I from the current study aimed to evaluate whether the test diet would give acceptable growth performance in comparison to the commercial diets locally used for rearing cobia, and thus may be suitable to be used when designing further experiments to assess requirement of amino acids in this species. Findings from experiment I indicate that juvenile cobia tolerate diets with low fishmeal inclusion provided that dietary amino acid profiles are balanced towards the anticipated requirements. Therefore, the plant-based protein formulation as used in the present experiment is as a good starting point for further requirement studies in this species and for producing a low fishmeal-based diet for cobia farming.

5.3 Lysine to arginine ratios on plasma free amino acids

The concentrations of plasma FAAs in cobia at 24 h postprandial in the current study (Table 3; Paper I) compares well to that reported for cobia in a previous study in which the fish were fed different levels of lizardfish silage (Mach and Nortvedt 2011). Plant ingredients, e.g. wheat

gluten or maize gluten may influence the retention efficiency of crystalline lysine that has been reported in rainbow trout (Tran *et al.* 2007). In addition, added crystalline amino acids in the diet may probably be absorbed more quickly than protein bound amino acids released from the digestion of soybean protein (Ambardekar *et al.* 2009). The asynchrony of FAAs released from diets for absorption could reduce the efficiency of protein synthesis if added crystalline lysine and arginine are absorbed and catabolized before the protein bound amino acids are released. This asynchronization will reflect in the pattern of changes in plasma FAAs. Generally, concentrations of plasma FAAs increase and peak following feed intake, and then return to the pre-feeding levels (Ambardekar *et al.* 2009, Karlsson *et al.* 2006, Murai *et al.* 1987, Thebault 1985). The time point that most of the amino acid are assumed to return to the pre-feeding levels in cobia was about 24 h (Mach and Nortvedt 2011). Had the inclusion of the plant ingredients and added crystalline lysine and arginine in the BL/A diet caused negative effects on absorption and utilization of amino acids, cobia fed this diet would require longer time for digestion and absorption compared to cobia fed the CD1- and CD2 diet. If this was the case, higher concentrations of plasma FAAs would be expected in cobia fed the BL/A diet, compared to cobia fed the CD1- and CD2 diet at 24 h postprandial. However, concentrations of plasma FAAs in cobia fed the BL/A diet were similar to cobia fed both the CD1- and CD2 diet 24 h postprandial, and comparable to that reported by Mach and Nortvedt (2011). These results suggested that cobia had utilized well the plant-based protein test diet as well as both the CD1- and CD2- diet in the current study.

Concentrations of plasma FAAs in cobia at 5 h postprandial (Table 5; Paper II) were also in accordance with that in cobia reported by Mach and Nortvedt (2011) that plasma indispensable FAAs peaked within 6 and 12 h postprandial. Previous studies reported that plasma FAAs reach maximum concentration at different postprandial times, and vary depending on species as well as dietary composition, digestion, absorption and the postprandial blood sampling time chosen (Espe *et al.* 1993, Espe *et al.* 2006, Plakas *et al.* 1980). In common carp (Plakas *et al.* 1980) and Nile tilapia (Yamada *et al.* 1982) fed casein-based diets, the peaks for most plasma indispensable FAAs occurred at 4 h postprandial. Also the solubility of the dietary protein affects the postprandial peak of plasma indispensable FAAs. Rainbow trout fed a diet with casein-based protein had highest levels of plasma FAAs between 12–36 h postprandial, compared to between 4-12 h in fish fed a diet in which the dietary protein was replaced by crystalline amino acids (Murai *et al.* 1987, Schuhmacher *et al.* 1995, Walton *et al.* 1986, Yamada *et al.* 1981). Compared to these, Atlantic salmon fed diets containing hydrolyzed protein or casein or fishmeal peaked 6 h postprandial (Espe *et al.* 1993, Sunde *et al.* 2003,

Torrissen *et al.* 1994). In the current study, blood samples were collected at 5 h postprandial suggested by Mach and Nortvedt (2011) that most plasma indispensable FAAs peaked at around 6 h post prandial. Plasma FAAs of cobia did not reflect dietary lysine and arginine may due to the fact that amino acids absorbed from the GI-tract are transported through the hepatic portal vein (HPV) to the liver, where they are catabolized. Thus, concentrations of plasma FAAs in blood taken from the HPV or dorsal aorta (DA) probably would reflect the absorption patterns of AAs better than blood collected from efferent branchial arteries that was done in this study. By comparing plasma FAAs collected from DA and HPV of adult rainbow trout, Karlsson and coworkers reported that FAAs have undergone hepatic modification during the first passed-through the liver (Karlsson *et al.* 2006). The authors also suggested a combination of both sampling cites for precise estimation of tissue assimilation and catabolism of a specific nutrient during digestion. However, to collect blood samples from HPV in larvae and juvenile requires sophisticated techniques and equipment that were not available in the current study. To confirm this further studies with such blood sampling techniques as well as with timed sampling are necessary.

Plasma FAAs did not reflected dietary lysine and arginine ratios may also be due to the fact that excessively absorbed AAs from GI-tract are transported to the liver, where the AAs will be either delivered to muscles for protein turnover or catabolizing for synthesis of other nitrogen products (e.g. nucleotides, nitric oxide), and for fuel. The major proportion of the limiting AAs will be used for protein synthesis, while AAs above the requirement will be oxidized rapidly (Anderson *et al.* 1993, Berge *et al.* 2002, Gahl *et al.* 1996, Walton *et al.* 1984). Carnivorous fish species may be able to deal with excessive dietary indispensable amino acids (i.e. 20% above the requirement, Harper *et al.* 1970) by maximizing capacity of catabolizing enzymes for the amino acids in questions (Berge *et al.* 2002, Walton *et al.* 1986). In an *in vitro* study on Atlantic salmon, Berge and co-workers reported that lysine acted both as a stimulant or inhibitor of the uptake of arginine depending on their relative concentrations, while excess arginine inhibited lysine uptake regardless of lysine concentration (Berge *et al.* 1999a). These authors also reported that dietary high arginine and lysine was reflected in plasma, muscle and liver of Atlantic salmon at 5 h postprandial. While, another study in Atlantic salmon showed that increased dietary lysine in diets adequate of arginine did not reflect the concentrations of these two amino acids in plasma, liver or muscle (Berge *et al.* 2002). In addition, plasma concentration of arginine and lysine in juvenile hybrid striped bass, *Monroe saxatilis x Chrysops* (Griffin *et al.* 1994) or in rainbow trout (Kaushik and Fauconneau 1984) did not seem to reflect the dietary levels of these two amino acids. Our findings were basically in line

with results reported by Kaushik and Fauconneau (1984), Griffin *et al.* (1994) and Berge *et al.* (2002) that when cobia fed dietary lysine and arginine above the requirement, concentration of these two amino acids in plasma would not reflect their dietary concentration. Results from the current study suggested that cobia may have adapted to excessive dietary lysine or arginine by increased catabolism for these two amino acids in order to control the balance of plasma FAAs. To confirm this, further studies on the intermediate metabolic products of lysine and arginine at post-absorptive levels are required.

It is also worth to emphasize that fish may show differences in assimilation efficiencies to different dietary amino acids (Rojas-Garcia and Rønnestad 2003, Rønnestad *et al.* 2001, Rønnestad *et al.* 2007). In golden perch, *Macquaria ambigua*, IAAs were generally absorbed at a slower rate than DAA (Anderson and Braley 1993). While, in an *in-vitro* study using the inverted intestine immersed in solutions of hydrolyzed casein, Rosas and coworkers reported that IAAs were absorbed at higher rates than DAAs in rainbow trout, totoaba, *Totoaba macdonaldi* and Pacific bluefin tuna *Thunnus orientalis* (Rosas *et al.* 2008). These inconsistence results suggest that amino acid absorption is species specific. It may be possible that some proportion of added lysine and arginine from the BL/A-, HL/A- and LL/A diet had leaked with different rates throughout the GI-tract via feces. Thus a reflection of these two dietary AA in plasma FAAs was not detected in the current study. However, nutrient markers, e.g. labeled-amino acids, were not used in the current study, therefore, whether there was such leakage of added free amino acids via GT-tract is still not conclusive. Further studies using the nutrient markers with timed sampling are necessary to elucidate absorption of lysine and arginine, and catabolism of these two amino acids in cobia.

In summary, results on the plasma FAAs at both 5 h and 24 h postprandial indicate that cobia can utilize the plant-based protein diets well as compared to the two commercial diets. Further studies are required to acquire knowledge on the catabolism of lysine and arginine at absorptive and post-absorptive levels in cobia.

5.4 Lysine to arginine ratios on growth, and deposition of protein and lipid

In the current study, the two commercial diets (CD1 and CD2) differed from three plant- based protein test diets (BL/A, HL/A and LL/A) in many aspects, including composition and protein and lipid contents as stated above (section 5.2). In the two plant-based protein test diets with imbalanced lysine to arginine ratios (LL/A and HL/A), one of the two AAs was kept at the same as its level in the BL/A diet and fulfilled the requirement (Ren *et al.* 2012, Zhou *et al.* 2007),

while the other AA was excessive. Therefore, discussion on the impact of different dietary lysine to arginine ratios on growth and deposition thereafter in this section is mainly based on the comparisons between cobia fed the BL/A-, HL/A- and LL/A diet (Paper II).

Juvenile cobia fed both of the HL/A- and LL/A diet showed reduced performance, compared to cobia fed the BL/A diet. Poorer growth performance, protein gain and/or PER of cobia fed the imbalanced lysine to arginine ratio diets (Table 3 and 4; Paper II) may be as a negative consequence of dietary excess of the two AAs. Fish and mammals used dietary AAs for protein turnover and synthesizing required biological products, while AAs above the requirement will be oxidized and excreted via urea or ammonium products, rather than being used for protein synthesis and growth (Anderson *et al.* 1993, Berge *et al.* 2002, Gahl *et al.* 1996, Walton *et al.* 1984, Zhou *et al.* 2011a). Nitrogen excretion is an energy-cost process. Impaired growth in fish fed excessive AA may due to the toxic effects (Choo *et al.* 1991) and stress caused by excess amount of the AA in the body of the fish leading to extra energy expenditure toward deamination and nitrogen excretion (Fournier *et al.* 2003, Tulli *et al.* 2007, Walton 1985). In addition, reduced growth in fish fed dietary excessive AA may be as a negative consequence of the accumulation of the AA or its degraded products in the body pools that could negatively affect enzymatic systems in fish, and further leads to accumulation and possible toxicity (Alam *et al.* 2002a). Dietary excessive arginine has also been reported to cause somatotropic effects on fish growth performance (Cho *et al.* 1992, Klein and Halver 1970, Lall *et al.* 1994, Luzzana *et al.* 1998, Walton *et al.* 1986). Previous studies have showed that excessive level of arginine in the diets may have adverse effects on weight gain, feed efficiency and/or PER in teleosts, including Nile tilapia, Indian major carp, hybrid clarias, grouper, *Epinephelus coioides* and rohu (Abidi and Khan 2009, Ahmed and Khan 2004a, Luo *et al.* 2007, Santiago and Lovell 1988, Singh and Khan 2007). Feeding diets with dietary lysine higher than requirement levels do not improve growth performance and feed utilization (Forster and Ogata 1998, Grisdale-Helland *et al.* 2011, Luo *et al.* 2006, Mai *et al.* 2006, Ruchimat *et al.* 1997), but rather reduce growth and feed efficiencies (Ahmed and Khan 2004b, Choo *et al.* 1991, Davies *et al.* 1997, Deng *et al.* 2010). Findings from the current study confirm those reported previously (Ren *et al.* 2012, Zhou *et al.* 2007), that growth performance in juvenile cobia was impaired when fed dietary excessive lysine or arginine.

Another possible explanation for the reduced growth and feed utilization in cobia fed the HL/A- and LL/A diet is due to the interaction between lysine and arginine. Lysine and arginine are assumed to share and/or compete for the same trans-membrane carrier systems including those

found in the intestine and kidney (Closs *et al.* 2004, Cynober *et al.* 1995). Antagonisms of the two amino acids may also reduce availability of the amino acids in tissues and thus reduce growth in rats and chick (Bedford *et al.* 1987, Fico *et al.* 1982, Harper *et al.* 1970, Jones *et al.* 1967, Nesheim 1968). Such interactions are known to impair growth in various mammals, for example in rats (Jones *et al.* 1966), cats (Anderson *et al.* 1979) and growing dogs (Czarnecki *et al.* 1985). In fish, however, the mechanism for the lysine-arginine interactions has not been unraveled as dietary imbalanced lysine to arginine ratios have shown inconsistent results (NRC 2011). Disproportionate dietary lysine-arginine levels caused no negative effects on growth and feed utilization in channel catfish (Robinson *et al.* 1981), red drum (Brown *et al.* 1988), rainbow trout (Kim *et al.* 1992), hybrid striped bass (Griffin *et al.* 1994), European seabass (Tibaldi *et al.* 1994), striped bass (Small and Soares 2000), Japanese flounder (Alam *et al.* 2002b) or Indian catfish, *Heteropneustes fossilis* (Ahmed 2012). On the other hand, imbalanced dietary lysine-arginine intake may suppress growth and feed utilization in rainbow trout as a result of the antagonism of the two amino acids at the level of ureagenesis (Kaushik and Fauconneau 1984), and a competition for absorption at the intestinal level (Kaushik *et al.* 1988). Similar results have also been found in Nile tilapia (Santiago and Lovell 1988), milkfish (Borlongan 1991), Atlantic salmon (Berge *et al.* 1997; 1998; 2002) and blackhead seabream, *Acanthopagrus schlegelii* (Zhou *et al.* 2011a). Increasing dietary lysine levels with adequate arginine did not cause significant effect on PER or daily protein retention in Atlantic salmon (Berge *et al.* 2002), while supplementation of arginine would partly ameliorate the reduced growth performance and feed efficiencies due to excessive dietary lysine in blackhead seabream (Zhou *et al.* 2011a). In the current study, the LL/A diet contained lysine at the requirement level, but excess arginine; while the HL/A contained excessive lysine, but arginine was kept at the requirement level. These two diets impaired growth and feed utilization in cobia, compared to the balanced lysine to arginine ratio diet suggested the possibility of similar antagonistic effect of excess dietary lysine on arginine availability. Further studies on utilization and interactions at absorptive and post-absorptive levels of lysine and arginine in cobia are required to acquire such knowledge.

In the present study, cobia fed the CD2 diet that had lysine to arginine ratio of 1.55 showed growth performance comparable to cobia fed the BL/A diet and better than the those cobia fed the HL/A- and LL/A diets (Paper II). Better growth performance in cobia fed the CD2 diet compared to the LL/A- and HL/A diets may due to the fact that this commercial diet contained higher taurine, OH-pro and methionine (Zhou *et al.* 2006) than the test diets. There was thus a possibility that cobia could have adapted to a certain level of disproportionate of lysine to arginine in the diets. This needs further studies to clarify. However, our findings support the

hypothesis that disproportionate dietary lysine-arginine intake causes negative impact on growth performance of juvenile cobia. Further studies are required to elucidate the possibility of such antagonistic relationship as well as the mechanism for lysine-arginine interactions in this species are needed.

Cobia fed the BL/A diet showed condition factor (CF), viscerosomatic index (VSI) and hepatosomatic index (HSI) comparable to those cobia fed the CD2 diet (Table 3; Paper II), while cobia fed the LL/A diet showed lower CF and were leaner than those fed the CD2 diet. However, CF and VSI were not different between cobia fed any of the LL/A-, BL/A- and HL/A diets, but higher HSI in cobia fed the LL/A- and HL/A diets, compared to those fed the BL/A diet. As liver contained high level of lipid, relatively bigger liver size indicated that more energy had been signaled to lipid deposition in cobia fed the LL/A- and HL/A diets. Previous studies reported that HSI in European seabass, rainbow trout and yellow grouper, *Epinephelus awoara* tended to increase when the fish were fed diet below the requirement of arginine, but fish fed dietary arginine above the requirement did not differ from those fed diet with arginine at the requirement levels (Riley *et al.* 1996, Tibaldi *et al.* 1994, Tulli *et al.* 2007, Zhou *et al.* 2012). In largemouth bass, *Micropterus salmoides* dietary arginine did not influence CF, VSI and HSI of the fish (Zhou *et al.* 2011b). Dietary lysine has been reported not to reduce HSI in European seabass, *Dicentrarchus labrax* (Tibaldi *et al.* 1994), Asian seabass, *Lates calcarifer* (Murillo-Gurrea *et al.* 2001), gilthead seabream (Marcouli *et al.* 2006) and blackhead seabream (Zhou *et al.* 2011a), while Atlantic salmon showed increased HSI when fed diets with lysine and/or arginine below requirement levels (Berge *et al.* 2002, Espe *et al.* 2007). Dietary excessive lysine has also been reported not to affect HSI, VSI and CF in blackhead seabream, cobia or pacu (Bicudo *et al.* 2009, Zhou *et al.* 2007, Zhou *et al.* 2010). In addition, dietary disproportionate lysine-arginine did not affect CF and/or VSI in Atlantic salmon (Berge *et al.* 2002) or blackhead seabream (Zhou *et al.* 2011a). Findings from the current study are basically in lines with previous studies mentioned above that disproportionate lysine-arginine did not affect CF and VSI, but increased HSI in cobia.

In the current study, protein and lipid content in the muscle, liver and whole body were not different between cobia fed any of the LL/A-, BL/A or HL/A diets. Previous studies in Indian major carp, hybrid clarias and blackhead seabream showed that protein content in the whole body of the fish increased with increasing dietary arginine and decreased when dietary arginine was higher than the requirement (Ahmed and Khan 2004a, Singh and Khan 2007, Zhou *et al.* 2010). Fish often show increased protein and decreased lipid deposition when dietary lysine and

arginine increase towards the requirement, but this is not the case when dietary lysine and arginine increase further. For instances, dietary arginine did not affect protein and lipid content of the whole-body composition in grouper, while increasing level of dietary arginine towards the requirement resulted in increased protein content of muscle and liver of the fish, but the lipid content decreased (Luo *et al.* 2007). However, when dietary arginine increased above the requirement, no differences in protein and lipid content of muscle and liver were detected (Luo *et al.* 2007). Similarly, dietary lysine above the requirements did not affect protein and lipid content of the whole-body composition in pacu (Bicudo *et al.* 2009). Further, disproportionate dietary lysine-arginine did not influence protein and lipid content of the whole-body blackhead seabream (Zhou *et al.* 2011a). Findings from the current study were in accordance with previous studies by Zhou *et al.* (2007) in cobia that dietary arginine or lysine above the requirement did not significantly affect protein and lipid content of the muscle, whole-body and/or liver of the fish.

5.5 Lysine to arginine ratios on appetite, feed intake and brain npy *and* cck *expression*

Lysine to arginine ratios and appetite and feed intake

In aquaculture, understanding the impact of dietary amino acids on appetite and feed intake and the mechanism controlling feeding behavior in fish is of vital importance. This will help to establish more accurate amino acid requirements and formulate diets giving better growth and feed efficiencies. In the current study, juvenile cobia fed disproportionate dietary lysine to arginine ratios showed reduced daily feed intake compared to those fish fed the balanced lysine to arginine ratio diet, and thus poorer FCR than those fed the BL/A and CD2 diet (Table 4, Paper 2; Fig. 4, Paper 3). These results indicate that dietary lysine to arginine ratios affect the appetite in cobia that consequently reduces the growth. Indispensable amino acids are known to being involved in the control of appetite and feed intake in fish (Gloaguen *et al.* 2012, MacKenzie *et al.* 1998). As in addition to protein turnover, IAAs and their metabolic products function as biological messenger molecules acting on intercellular signaling pathways (Morley and Flood 1991). Alternation of IAAs may result in pronounced effects on neuro-endocrine systems regulating appetite and feeding behavior (MacKenzie *et al.* 1998). Excessive or imbalanced dietary IAAs ingested by animals not only cause reduced feed intake, and thus retardation of growth but also cause pathological lesions and even death (Harper *et al.* 1970). Rats showed significantly decreased feed intake within the first bout of feeding and complete rejection in the following meals when offered diets that were devoid of a single indispensable amino acids (Koehnle *et al.* 2003). Feeding dietary deficiency of valine reduced feed intake in pigs, while this negative effect was more severe when dietary leucine was supplied in excess of

the requirement (Gloaguen *et al.* 2012). Similar results were reported in rainbow trout that the fish showed apparent aversion to lysine-devoid diet, and was able to select against lysine-deficient diet, suggested that rainbow trout discriminate sufficiency of lysine in diets and regulate their feed consumption (Yamamoto *et al.* 2001). Also, the striped bass, *Morone saxatilis* (Hughes 1997), Indian major carp (Murthy and Varghese 1998) as well as the black sea bream (Zhou *et al.* 2011a) showed reduced feed intake when fed disproportionate amino acid diets. Deficiency of dietary lysine and arginine or disproportionate dietary lysine to arginine ratios are also known to reduce feed intake and/or feed utilization in milkfish, common carp, rainbow trout, Atlantic salmon, Indian major carp, Japanese seabass and rohu (Abidi and Khan 2009, Ahmed and Khan 2004a, Berge *et al.* 2002, Borlongan and Benitez 1990, Davies *et al.* 1997, Mai *et al.* 2006, Tacon 1992). The results from the current study confirm the hypothesis that imbalanced dietary lysine to arginine ratios cause reduced appetite and feed intake in juvenile cobia, and that this consequently reduce growth.

Dietary indispensable amino acids play important role in growth during the early life stages of fish (Rønnestad *et al.* 1999, Rønnestad *et al.* 2003). Imbalances of dietary amino acids often result in increased oxidation of amino acids and decreased feed conversion efficiencies and subsequently, reduced growth in fish and mammals (Gomez-Requeni *et al.* 2003). Though, the capacity to cope with imbalanced dietary IAAs in fish may differ from that in mammals. Fish may be able to increase oxidation of amino acids from an imbalanced diet, converting to ammonia and urea for excretion. Further, fish often show a higher plasma ammonia tolerance and level compared to that of mammals (Wright 1995), thus they would tolerate the imbalanced dietary IAAs better than mammals (Dabrowski *et al.* 2007). However, the results from the current study indicate that imbalanced lysine to arginine ratios reduced appetite and feed intake in cobia, suggesting that lysine or arginine may have a role in the regulation of feeding behavior of this species. The mechanism and signaling pathways behind these observations should be investigated in further studies.

Feeding status and dietary lysine to arginine ratios on the expression of npy *and* cck

Data from literatures show that NPY and CCK are highly conserved throughout evolution (Blomqvist *et al.* 1992, Crawley and Corwin 1994, Larhammar 1996, Pedrazzini *et al.* 2003). This suggests that these two neuropeptides have a similar vital role in regulations of physiological processes among fish as in mammals, both teleosts and cartilaginous fish (MacDonald and Volkoff 2009). The current study was the first attempt to clone the cDNA for NPY and CCK in cobia in order to target their role in signaling pathways controlling appetite

and feeding behavior. Results from the current study indicate that amino acid sequences of NPY and CCK in cobia show high homology to that observed in other fish (Fig. 1 and Fig. 2, Paper III). These findings provide additional evidence that these two peptides are highly conserved among fish (MacDonald and Volkoff 2009).

npy *expression*

In the current study, for most of the diets (BL/A-, HL/A and CD2), the unfed cobia (i.e. before a meal) had a higher expression of *npy* in the brain, compared to fed cobia (i.e. after a meal). Meanwhile, no significant differences in brain *npy* expression were observed between cobia fed diets with different lysine to arginine ratios (Fig. 6 and Fig. 8, Paper III). Changes of NPY levels (both mRNA and peptide), with an increased levels prior to a meal and a fall after a meal have been reported in rats (Kalra *et al.* 1991, Sahu *et al.* 1992) and in goldfish (Narnaware *et al.* 2000). Goldfish showed increased brain *npy* expression at 1-3 h before the mealtime (Narnaware *et al.* 2000), while brain *npy* level in Atlantic cod was higher at mealtime (0 h) than 2 h before the meal (-2 h) (Kehoe and Volkoff 2007). Similar results were reported in Brazilian flounder that brain *npy* level was higher at mealtime (0 h), compared to at 2 h before mealtime (-2 h) and 2 h after feed intake (Campos *et al.* 2012). The findings from the current study are in line with the above mentioned studies, and support that NPY has a role in appetite regulation during a meal in cobia, although it is difficult to find a clear link with dietary contents of lysine and arginine.

After short-term feeding (week 1), brain *npy* level in cobia fed the LL/A diet did not differ from pre-feeding cobia. Cobia fed this diet also had less stomach filling compared to cobia fed any of the other diets (Fig. 4, Paper III). These results suggested that at this sampling time cobia fed the LL/A may be still hungry but a loss of appetite had led cobia to avoid feeding. These were not the case after long-term feeding (week 6). After six weeks of feeding, the situation was different that results in *npy* expression in cobia fed the LL/A diet were comparable to that observed for cobia fed the other three diets. At week 6, all groups had a higher expression of *npy* in unfed compared to fed cobia. Also, the stomach filling were comparable for all diets (Fig. 5, Paper III). This suggests that cobia had adapted to the LL/A diet after long-term feeding. Our findings also suggest that brain *npy* had a faster response in term of expression following a meal when cobia had been offered the imbalanced lysine to arginine ratio diet (i.e. the LL/A diet) for long-term feeding, compared to short-term feeding.

It is possible that the lack of significant differences in *npy* expression between unfed cobia and fed cobia the LL/A diet in the samples one week into the feeding may be due to the time that was selected for sampling (15 minutes after feed ingested). Following the initial ingestion of food in a meal, the GI-tract and associated organs will gradually secrete a range of substances that contribute to finally terminate the meal. These satiety signals will be integrated into the regulatory pathways in the hypothalamus. Further, there may be also dynamic changes in the brain levels of NPY during the course of a meal, that the levels of *npy* are progressively reduced at the end and after a meal in response to the satiety signals from the GI-tract. In addition, periprandial changes in the expression of *npy* differ between different areas of the fish's brain. For instance, goldfish showed increased in *npy* levels in the telencephalon-preoptic area and hypothalamus shortly before feeding, while *npy* levels decreased in optic tectum-thalamus (Narnaware *et al.* 2000). In the current study, the sampling time was only 15 minutes after feeding, and *npy* of whole brain was analyzed. These two factors could be possible explanations for not detecting a significant drop in cobia fed the LL/A diet at week one. However, further studies with regional expression analysis and time sampling periprandial are necessary to acquire knowledge on the patterns of changes in the expression of *npy* in cobia.

In summary, results from our study indicate that low lysine to arginine ratio in the diet reduced appetite and affected the expression of brain *npy* of cobia in the first week after the diets were introduced. The general patterns of changes in the levels of brain *npy,* with significant lower levels after feeding were observed among cobia fed the LL/A diet at week six, and cobia fed the other three diets both at week one and at week six suggest that NPY serves as a hunger factor in cobia. These results also suggested that there may exist a mechanism regulating appetite-adaptation and feeding behavior to dietary arginine to lysine ratios in cobia.

cck *expression*

Results from Paper III did not indicate that *cck* expressed in the brain is an important player in appetite regulation, as there were no significant differences in brain *cck* levels between unfed and fed cobia. The four diets did not differ from unfed cobia, either at week one or at week six (Fig. 7 and Fig. 9). In goldfish, brain *cck* levels increased 2 h postprandial (Peyon *et al.* 1999). Increased expression levels of both *cck-l* and *cck-n* from the GI-tract were observed within 1.5 hours after a meal in Atlantic salmon (Rønnestad *et al.* 2010). While, in another study on Atlantic salmon, the fish showed different patterns of changes in expression of different isoforms of brain *cck* postprandial; *cck-l* level was elevated within 12 h postprandial, while the change in *cck-n* level was not significant (Valen *et al.* 2011). The lack of significant changes in

brain levels of *cck* in cobia may be due to the fact that any change in *cck* expression after a meal may take some time. Given that cobia were sampled 15 minutes after the meal, short-term changes in *cck* expression could have been missed in the current study. More studies with timed sampling to elucidate the patterns of changes in brain *cck* are needed.

Differences in brain *cck* were not detected between fed and unfed cobia from our study may be due to the fact that CCK-producing cells are very differently distributed from organs to organs (e.g. brain parts and GI-tract) (Rønnestad *et al.* 2007). However, the response of *cck* following a meal may be related to the role(s) of CCK in the respective organs. In the GI-tract, CCK plays a range of roles in the regulation of the digestive response to a meal, of which satiation signaling to the brain is only one, and the multiple roles of CCK in the brain signaling added to this complexity. Regional expression of *cck* may also vary from species to species. In goldfish brain, the highest levels of *cck* expression were detected in hypothalamic region compared to other regions such as olfactory bulbs, telencephalon and preoptic region, optic tectum–thalamus and posterior brain regions (Peyon *et al.* 1999). Rainbow trout differed in the expression of *cck* isoforms in the brain and the GI-tract, of which *cck-n* was mainly expressed in the optic tectum, but of *cck-l* highly expressed in the saccus vasculosus and part of the hypothalamus (Jensen *et al.* 2001). Meanwhile, *cck* expressesed mainly in the anterior intestine in yellowtail (Murashita *et al.* 2006). In the current study, we were unable to differentiate the brain of juvenile cobia into different regions; thus we could not ascertain whether there were regional differences in changes in brain *cck* levels. Further studies with regional expression analysis to elaborate the expression of *cck* are required.

As previously mentioned the regulation of feeding behavior and food consumption in fish takes place in the brain with multitude of orexigenic and anorexigenic neuropeptides, neurotransmitters and hormones released in the central nervous system and peripheral tissues (Volkoff *et al.* 2010). Generally, orexigenic factors progressively elevate before to the mealtime in accordance to the increase of hungry levels and are reduced during and following feeding. The anorexigenic factors are stimulated and increase in response to the fullness of the GI-tract or levels of satiety during and following feed consumption. In general, NPY is considered the most potential orexigenic signal molecule, while CCK acts as a satiety factor. If this is the case in cobia, it would be expected that pre-feeding (or unfed) cobia has higher brain *npy*/NPY levels, but lower *cck*/CCK than in fed satiety cobia following a single meal. The findings from the current study support the idea that NPY is involved in the regulation of feeding behavior,

and functions as an orxigenic signal in cobia, while the role of CCK in the regulation of feeding behavior in this species is not yet conclusive.

In the current study the role of NPY and CCK in feeding behavior were indirectly targeted via measuring their brain expression levels. In order to have a better understanding on the impact of dietary imbalanced amino acids and the role of NPY and CCK in the regulation of feeding behavior in cobia, characterization and localization of these two peptides by using *in situ* hybridization technique are suggested. Furthermore, NPY and CCK may also play several physiological roles in cobia. As other neuropeptides, NPY and CCK may transmit the signal by acting on their receptors, thus further studies on NPY- and CCK- receptors gene using receptor antagonists or more sophisticated peptide knockdown and/or gene knockdown are required to understand the mechanism of their interactions as well as the role of these peptides in cobia. In addition, feeding behavior is regulated by a multitude of orexigenic and anorexigenic factors. In order to have better understanding on the impact of dietary imbalanced amino acids on appetite and feed intake in cobia, further studies on NPY and CCK in association with other orexignic and anorexigenic factors, e.g. neuropeptide YY, pancreas peptide (PP), agouti-related peptide (AgRP), orexin (OX), cocain- and amphetamine-regulated transcript (CART), pro-opiomelanocortin (POMC), gastrin, leptin, etc. as well as their receptors in the signaling pathway circuit are required.

6. GENERAL CONCLUSIONS

Juvenile cobia had a high voluntary feed intake when offered the two commercial diets and the plant-based protein test diet. When fish were fed to satiation, most of the content in the stomach was emptied within 8 h post-feeding.

Juvenile cobia showed good growth performance and feeding efficiency when fed on diets with 730 g kg^{-1} plant ingredient inclusion; given that dietary amino acids fulfilled the nutritional requirements of the species and that the ratio of lysine to arginine was balanced. Plant protein inclusion did not affect appetite, growth and CF, VSI and HSI in cobia. This suggests that cobia tolerate moderate to high levels of plant ingredient inclusion. The test diets used in the present study may serve as a good starting point for studying nutrient requirements in cobia, and serve as a basis for commercial formulation diets with high plant ingredient inclusion.

Altering the dietary ratios of lysine to arginine affected appetite, feed efficiency and growth performance, and protein and lipid deposition in cobia. An imbalanced dietary lysine to arginine ratio led to reduced appetite and growth performance, but higher HSI in cobia.

Plasma lysine to arginine ratios analyzed at 5 and 24 h postprandially did not reflect dietary lysine to arginine ratios.

Juvenile cobia showed decreased brain expression of *npy* post feeding, suggesting that NPY is an orexigenic signal that is involved in the regulation of appetite and feeding behavior in this species.

There were no significant changes in brain *cck* expression pre-feeding and directly post-feeding, thus the role of CCK from the brain in the signal pathways controlling the appetite and feeding behavior in cobia remains unclear.

There were no clear correlations between the diet, feed intake, growth and gene expression of the neuropeptides involved in appetite regulation.

Data on *npy* expression indicated a long-term adaptation in the appetite response that was observed when measured after week 1 post-feeding and week 6 post-feeding the experimental diets. These results suggested a mechanism controlling appetite-adaptation in cobia may exist.

7. FUTURE PERSPECTIVES

This study was the first attempt to determine the impact of dietary balanced lysine (L) to arginine (A) ratio versus imbalanced dietary L/A ratios in high plant protein and low fishmeal inclusion diets for cobia. The results from the current study provide a good starting point for studying nutrient requirements in cobia and serve as a basis for commercial formulation diets with high plant ingredient inclusion for cobia.

Further studies using nutrient markers are required to totally elucidate the utilization of crystalline lysine and arginine as well as the antagonism (if any) at absorptive and post-absorptive levels and catabolism of these two AAs in cobia. To acquire knowledge on kinetics of plasma FAAs postprandial hepatic vein blood sample collection with time sampling is suggested.

In order to optimize the use of derived-plant proteins in the commercial pellets for juvenile cobia, further research on the impact of other dietary IAA (particularly the branch-chained and sulfur amino acids) with experimental design similar to that used in this study with more timed sampling are suggested.

Further studies to determine the optimum dietary AAs that will fulfill both metabolism and growth and deposition with acceptable feed utilization and feed efficiencies for different life stages under different environmental conditions are required.

Findings from this study indicate that NYP is involved in signal pathways that control the appetite and feeding behavior in cobia, while the role of CCK in such pathways is yet conclusive. Further, results on the expression of brain *npy* at week one and week six suggest an appetite-adaptation regulation for dietary amino acid ratios in cobia. However, further studies with regional expression of the two genes (*npy* and *cck*) with time sampling periprandial are necessary to totally elucidate orexigenic and anorexigenic effects of NPY and CCK.

More research is required to understand whether dietary amino acid composition affects mechanism of regulation of appetite adaptation and gene expression of *npy* and *cck* cobia. In order to acquire knowledge on the role of NPY and CCK in signal pathways that control appetite and feeding behavior in cobia, more studies with regional characterization of these two

neuropeptides and their receptor genes using *in situ* hybridization, knockdown of neuropeptides by receptor antagonists, and knock-out gene techniques are suggested.

Biological functions of NPY and CCK are presumed to be conserved in vertebrates, but their mechanism of actions may be species-specific and depend on season, food availability nutritional status, etc. Understanding of the functions of these two neuropeptides in cobia is absent. Therefore, further studies on the role of NPY and CCK in regulation of homeostatic, energy balance and hormonal secretion are required to fill this knowledge gap in cobia.

To understand the mechanism controlling appetite and feeding behavior in cobia, more studies on NPY and CCK in association with other orexignic and anorexigenic factors, e.g. neuropeptide YY, pancreas peptide (PP), agouti-related peptide (AgRP), orexin (OX), cocain- and amphetamine-regulated transcript (CART), pro-opiomelanocortin (POMC), gastrin, leptin, etc. together with their receptors in a signaling pathway circuit are required.

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